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ENDOMYCIN, A NEW ANTIBIOTIC[1]

DAVID GOTTLIEB, P. K. BHATTACHARYYA,
H. E. CARTER, AND H. W. ANDERSON[2]

(Accepted for publication November 30, 1950)

Of the many antibiotics reported within the past few years, very few have been antifungal agents (3, 6, 7, 9, 11, 12, 14, 15, 16, 17, 18), and none of these have as yet been accepted as therapeutic agents for fungal diseases. Our experience indicates such fungus inhibitors are produced by many actinomycetes, though not so commonly as are the bacterial inhibitors. If a survey is directed toward finding organisms antagonistic to the fungi responsible for animal mycoses, the number of active agents is even more reduced. One such actinomycete, isolate 9-20, obtained from soil plates, possesses antifungal and antibacterial properties. A new antibiotic, endomycin, has been isolated from this organism; it prevents the growth of a large number of fungi and some bacteria. Two other materials were also produced by the organism but these have not been characterized chemically.

THE ORGANISM

Isolate 9-20 was obtained from soil collected in the Cache River Valley of Illinois and was chosen for further study on the basis of screening procedures described in a previous paper (8).

The morphology, development, and growth of the organism were studied on a large number of media, and detailed observations of sporulation were made on growing cultures in depression slides. The isolate was not identical with any of the Actinomycetaceae as described in Bergey's Manual of Determinative Bacteriology (2). The absence of pigment formation in the medium would indicate its relationship with *Streptomyces albus*, but other characteristics differentiate isolate 9-20 and *S. albus*. Streptomyces 9-20 hydrolyzes starch, does not peptize litmus milk after coagulation, produces an acid reaction, and does not readily liquefy gelatin; the converse properties are attributed to *S. albus*.

In a favorable medium, a base mycelium is first formed which has no distinct color of its own but which takes on the color of the agar medium and consists of rather straight ramifying hyphae. A white aerial mycelium develops on the third day and gives rise to the sporophores which are formed along the entire length of the parent mycelium and are at right angles to it. Soon a sporophore bends slightly, then twists and coils in a spiral; it increases in diameter and begins to divide into spores. As this process continues, the spores darken and the spiral becomes very compact, resembling a coiled spring, often with ten loops so tightly coiled that when the spiral is

[1] This investigation was supported by grants from the Abbott Laboratories, Eli Lilly and Company, and the Upjohn Company to the Departments of Horticulture and Chemistry of the University of Illinois.

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broken, each turn resembles a doughnut. Concomitantly with sporulation, the white surface of the colony becomes light gray and later dark gray. Sporulation begins in the older parts of the colony while the younger portions on the periphery are actively growing. In old cultures the substrate mycelium turns black and disintegrates. The peripheral hyphae vary from 0.7 to 1.0 μ in diameter; older hyphae are thicker, 1.25 to 1.5 μ. The spirals are about 3.5 to 4.0 μ in diameter with an open center of about 1.4 μ. The spores are not readily detached and tend to adhere to the spiral form.

The following carbohydrates are readily utilized when included in Czapek's alkali medium containing no other source of carbon: starch, mannose, dextrin, glucose, arabinose, maltose, levulose, gelatin, and asparagin. A number of carbon compounds supported only poor growth: galactose, lactose, citric acid, succinic acid, and malic acid. Cellulose was also a poor carbon source, for few small colonies developed. No growth or at most only faint traces occurred on sucrose, sorbitol, dulcitol, inositol, or paraffin. Growth is poor on Czapek's acid agar with starch as a carbon source. Isolate 9-20 utilizes NH_4 as well as NO_3 nitrogen. It coagulates litmus milk but peptizes the protein only very slowly, if at all, and produces an acid reaction. Starch is readily hydrolyzed, but gelatin is slowly and only slightly liquefied.

Growth was good on potato slants and potato-dextrose agar, with heavy raised mycelium and a light gray surface. On Emerson's agar and nutrient agar growth was excellent, with early graying and sporulation. Tryptone glucose agar and North's gelatin agar supported good growth with white aerial mycelia, but not spiral formation, and consequently sporulation was absent. Only after 14 to 16 days did even slight graying occur on North's gelatin medium, and none occurred on Tryptone glucose agar. Isolate 9-20 never produced a soluble pigment in either the complex natural or the chemically defined media.

SPECTRUM

Streptomyces 9-20, when streaked on Petri plates, inhibited the growth of 35 species of fungi and 15 bacteria. None of the fungi, including 15 yeasts, were resistant to this streptomycete; the growth of two bacteria, *Escherichia coli* and *Serratia marcescens*, was only slightly inhibited, and *Pseudomonas aeruginosa* was unaffected. Especially interesting is the inhibiting effect of isolate 9-20 on all the plant pathogens which were tested as well as the human pathogens, *Trichophyton interdigitales*, *Blastomycoses dermatitidis*, and *Microsporium audouini*. A complete table of the streak spectrum is not included but all organisms that were inhibited by solid preparations of endomycin are in table 2.

PRODUCTION IN SHAKE FLASK

All shake-flask studies were made with 100 ml. of medium in 500-ml. Erlenmeyer flasks on a reciprocal shaker. The inoculum was prepared by

making a spore suspension in distilled water from a 7-day-old culture of *Streptomyces* 9-20 and adding the suspension medium in a flask. A 2-day-old shake culture of this medium was then used to inoculate the other flasks. In the early stages of this research the antibiotic production was determined by the agar-plate method of Loo et al. (13) on streptomycin assay plates and the diameter of the inhibition zone interpreted as equivalent streptomycin units (Table 1). Despite the ease with which *Streptomyces* 9-20 produces antibiotics in semisolid agar media, the original culture did not readily produce them in liquid media. Production improved after the organism had been transferred repeatedly, but even then the yields were erratic. With the addition of agar, antibiotic production in the brew increased as the concentration of agar was raised, but in practice the amount of agar was kept at 0.025 per cent to prevent gelation of the media. The greatest production of endomycin occurred in Abbott soybean[3] and Emerson media.[4] Later the organism was adapted to the soybean medium and produced endomycin in the absence of agar. Activity reached its peak be-

TABLE 1.—*Production of antibiotics in shake flasks*

Number of hours	Equivalent streptomycin units[a] in	
	Abbott medium	Abbott medium + agar
48	36	68
72	68	96
96	116	240
120	116	136
144	116	240
168	80	200

[a] Averages for three replicates.

tween the 4th and the 6th day after inoculation, then declined slightly, though after even 14 days the reduction was only 25 per cent (Table 1). Since the more significant activity of endomycin is against fungi, the brews of *Streptomyces* 9-20 were tested against the yeast *Torula utilis* by the dilution method. Usually a concentration of 60 to 80 dilution units per ml. of brew was obtained, though sometimes the organism produced as high as 120 units per ml. The germination of *Sclerotinia fructicola* spores was also inhibited by the shake-culture fluids, and on agar plates a 1:20 dilution prevented the growth of *Gliocladium fimbriatum*, *Microsporium canis*, *Rhizoctonia solani*, and *Fusarium lycopersici*.

Most of the endomycin is retained in the mycelium and only a small amount diffuses into the culture medium. From 21 l. of brew, 9,000,000 units were obtained, of which 7,000,000 were extracted from the mycelium and only 2,000,000 from the brew. Assuming 100 per cent recovery, the

[3] Soybean meal, 10.0 gm.; cerelose, 10.0 gm.; NaCl, 5.0 gm.; Curbay B.G., 0.5 gm.; $CaCO_3$, 1.0 gm.; distilled water, 1000 ml.

[4] Cerelose, 10.0 gm.; yeast extract, 1.0 gm.; sodium chloride, 2.5 gm.; beef extract, 4.0 gm.; Bactopeptone, 4.0 gm.; agar, 20.0 gm.; distilled water, 1000 ml.

organism produced 429 units per ml. of culture suspension, of which approximately 78 per cent was in the mycelium.

The antifungal factor in the culture fluid is stable to 100° C. for at least 30 min. and withstands autoclaving at 15 lb. for 20 min. It is also stable in wide ranges of hydrogen-ion concentration, since no inactivation occurs at pH 1.1 or pH 11.5 during 24 hr.

ASSAY

A unit of endomycin was defined as the minimum amount of antibiotic per ml. which would inhibit the growth of *Torula utilis* for 24 hr. in Emerson's medium. When the brew was assayed, the potency was expressed as units per ml. and was obtained by determining the greatest dilution of 1 ml. which would prevent the growth of the test organism. For solid preparations, the potency was recorded as units per mg., and the number of such units was equal to the greatest number of ml. in which 1 mg. could be dissolved and still stop the growth of *T. utilis*. A standard 3-mm. loop of a 16-hr. culture of *T. utilis* was used for inoculum in all cases.

EXTRACTION OF THE SALT OF ENDOMYCIN

Twenty-five liters of a 6-day-old culture of *Streptomyces* 9-20 containing 150 dilution units per ml. of culture fluid were separated into two components by centrifugation and decantation, 1) the mycelium and 2) the supernatant liquid, each of which was extracted separately. The mycelium was extracted with 8 l. of butanol using 1 l. each time. The combined butanol extracts, assaying 900 u./ml., were evaporated with addition of water until a brown gum remained. This residue was further extracted with 500 ml. of ether, in two operations, which removed most of the coloring matter and left a light brown powdery residue assaying about 1,300 u./mg. The total yield of solids was 4.6 gm. and the total recovered activity 6,000,000 units. Twenty-two liters of the supernatant liquid were extracted with 8 l. of butanol in two portions. These butanol extracts were washed with water, evaporated to a gum, and extracted with 500 ml. of boiling ether. From this procedure a brown residue of 4.2 gm. was obtained assaying 500 u./mg. or 2,100,000 units.

Isolations of the free acid: One hundred and twenty-one grams of a butanol extract from 139 l. of brew was received from Dr. W. G. Jackson of the Upjohn Company; it assayed about 500 u./mg., or 60,500,000 units. This material was treated twice with 1-liter volumes of ether and finally with 300 ml. of ether by shaking the suspension for 2 to 3 hr. each time. The final centrifugate was then extracted with boiling benzene: 52.1 gm. of a light brown, fluffy residue contained 1,250 to 1,500 u./mg., or 60,150,000 units in all. When this material was suspended in 1.5 l. of water with vigorous stirring for 4 to 5 hr., a turbid aqueous emulsion was formed. This emulsion was then washed with ether until the ethereal extracts were colorless. The aqueous layer was next centrifuged, depositing about 0.30 gm. of

a white precipitate. Acidification of the still turbid supernatant liquid to pH 4.5 gave a dark brown, gummy substance containing most of the active material; it was allowed to settle overnight and the supernatant liquid was then decanted. The residue was taken up in 680 ml. of about 0.05 N. sodium hydroxide (final pH 8.4) with shaking and a small amount of residual solid removed. Twelve ml. of glacial acetic acid was next added to the clear supernatant portion, causing the precipitation of the antibiotic. This acid was separated from the liquor and dried to an amorphous solid over saturated KOH and P_2O_5. The yield of this solid was 32.89 gm. with an activity of 1,750 to 2,000 u./mg., or 57,557,500 units.

Eleven grams of this acid precipitate was purified further by dissolving it in 250 ml. of commercial absolute alcohol at room temperature for 4–5 hr. with shaking. The insoluble material was removed and the antibiotic reprecipitated with 8 volumes of ether. Three more reprecipitations from alcohol gave a product assaying about 4,000 units per mg.

The acid form of endomycin is soluble in alcohols, methyl cellosolve, and sparingly soluble in dioxane. It is insoluble in water, ether, chloroform, benzene, ethyl acetate, and other fat solvents.

Isolation of 9-20F-1. The extraction of *Streptomyces* 9-20 cultures with butyl alcohol removes another antibiotic different from endomycin. This material is separated from endomycin during the ether extraction by its solubility in that solvent. In different fermentations the yield of 9-20F-1 varies from a trace to 20 per cent. Its spectrum is very similar to that of endomycin, though more intensive studies might reveal different antibacterial properties.

Isolation of 9-20B. A third antibiotic is produced by *Streptomyces* 9-20 which can be separated on the basis of chemical and biological properties. This material is extracted by adsorption on Nuchar, elution with acid alcohol, and precipitation from that solution with ether. It is extremely hygroscopic, and in the crude state assays from 8 to 25 mycin units per mg. Antibiotic 9-20B is inactive against fungi and is thus different from the other compounds produced by the culture.

ANTIBIOTIC SPECTRUM OF ISOLATED ENDOMYCIN

Because all three antibiotics are probably produced in agar cultures of the actinomycete, the identity of extracted endomycin with the material produced in Petri plates could not be fully established. Nevertheless, the inhibitory properties of endomycin against fungi, Gram positive bacteria, and some Gram negative bacteria indicate its identity with antibiotic produced in the Petri plates. Some of the bacteria, such as *E. coli*, are inhibited on plates but not readily by solutions of endomycin; this discrepancy, however, can be attributed to the action of 9-20B on the streak plates. Endomycin inhibited the growth of all 15 fungi which were tested in broth culture (Table 2). It also inhibited nine bacteria at 10 units or less, and

two bacteria between 10 and 100 units (Table 2); three bacteria were not inhibited even at 100 units.

TOXICITY OF ENDOMYCIN[5]

Preliminary experiments indicate that endomycin is not highly toxic to mice. Two sets of three mice were injected with an aqueous suspension of the antibiotic. The three mice which received a dose of 500 mg. per Kg. body weight were in apparent good health 12 days after injection, whereas the three mice which received 1,000 mg. per Kg. died within two days.

TABLE 2.—*Bacteria and fungi inhibited by endomycin*

Microorganism[a]	Endomycin units[b] preventing growth	Microorganism[a]	Endomycin units preventing growth
Sclerotinia fructicola	1–5	*Bacillus cereus*	1–5
Torula utilis (various strains)	do	*Staphylococcus aureus*	do
Endomyces magnusii	do	*Corynebacterium xerose*	do
Mycoderma cereviseae	do	*Listerella monocytogenes*	do
Candida albicans	5	*Erysipelothrix rhusiopathiae*	do
Microsporium canis	7.5	*Brucella abortus* (strain 19)	do
Candida guilliermondi	25	*B. abortus*	do
Trichophyton interdigitales	do	*Bacillus subtilis*	5–10
T. rubrum	do	*Agrobacterium tumefaciens*	10
Microsporium audouini	do	*Mycobacterium tuberculosis* (strain 607)	10–100
Rhizoctonia solani	do	*Streptococcus faecalis*	do
Glomerella cingulata	do	*Aerobacter aerogenes*	> 100
Macrosporium sarcinaeforme	do	*Escherichia coli*	do
Colletotrichum phomoides	do	*Pseudomonas aeruginosa*	do
Fusarium lycopersici	25–50		

[a] Data supplied by Dr. Alma J. Whiffen of the Upjohn Company indicate that endomycin (Prep. III 30 III) inhibits nine additional fungi at concentrations of 10 μg./ml. or less; *Blastomycoses dermatitidis, Coccidioides immitis, Geotrichum* sp., *Hormodendrum compactum, Cryptococcus neoformans, Histoplasma capsulatum, Sporotrichum schenkii, Monosporium apiospermum, Microsporium audouini.*

[b] A unit is equal to the greatest number of ml. of broth in which 1 mg. of endomycin can be diluted and prevent the growth of a given strain of *Torula utilis* (Illinois 41).

BACTERISTATIC AND BACTERICIDAL ACTIVITY

Endomycin can prevent the growth of microorganisms in either of two ways, either by bacteristatic or by bactericidal action. *Candida albicans* and *Staphylococcus aureus* were inoculated into six concentrations of the antibiotic, 1, 5, 10, 50, 100, and 200 units per ml. One series of each was stored at 1° C. for 24 hr. to determine the effect of endomycin on cells which were not multiplying; the other series was kept at 37° C. for active growth. The organisms which were prevented from growing by low temperatures were apparently not injured by the antibiotic, for, when transferred to fresh broth, even the inoculum from the 200 unit tubes grew readily. When the organisms were incubated at 37° C., however, the effect on *C. albicans* was different from that on *S. aureus*. Growth of the yeast was inhibited in all concentrations above 5 units per ml., but none of the or-

[5] The results of these studies have been made available through the courtesy of Dr. G. F. Cartland of the Upjohn Company.

ganisms was viable in any transfers made from such tubes. Apparently, endomycin is fungicidal to *C. albicans* on long exposure. The effect on *S. aureus* is primarily bacteristatic. More than 5 u./ml. inhibited growth; nevertheless all transfers resumed growth when placed in a fresh medium, except that some retardation of growth was noticed in cultures transferred from 100 and 200 units per ml.

COMPARISON OF ENDOMYCIN WITH OTHER FUNGICIDES

Endomycin is different from other antibiotics active against fungi on the basis of either chemical or biological properties. It can be separated readily from viridin (5), glutinosin (6), actidione (18), and gladiolic acid (3) because these three antibiotics have very little antibacterial activity and are distinct in their chemical and solubility properties. Endomycin is not similar to gliotoxin (4, 15, 16) or clavacin (9, 10), since the latter compounds are soluble in chloroform and the former is insoluble in this reagent. Biologically, endomycin differs from them on the basis of its weak activity against *Escherichia coli*, whereas it is more active than clavacin against *Staphylococcus aureus* and of equal activity to gliotoxin against *Bacillus subtilis*. Streptothricin (17) is a basic, thermolabile material which is soluble in water and aqueous acid solutions; endomycin possesses the converse properties. Nor is this new antibiotic similar to antimycin (11, 12). The latter compound is unstable to alkali, is thermolabile, and is soluble in chloroform, ether, and benzene whereas none of these properties are attributes of endomycin. Of all the compounds endomycin seems most closely related to musarin (1, 14), but here again sufficient differences exist to show that they are not identical. The main differences are 1) musarin is unstable to heat whereas endomycin is stable, and 2) the methyl ester of musarin is inactive, that of endomycin being equally active with the parent material. Unfortunately, we were not able to obtain samples of musarin for direct comparisons. From these results, however, it is apparent that endomycin is a new antibiotic, unlike any previously described. Nevertheless, it is interesting to note that musarin and endomycin are both high molecular weight acids, form emulsions readily, and give poor assays on agar plates; both have a low nitrogen content (ca. 3.7 per cent).

SUMMARY

A new antibiotic, named endomycin, has been isolated from cultures of an unidentified species of Streptomyces. The antibiotic inhibits the growth of a wide variety of fungi pathogenic to plants and animals; and, in addition, is antibiotic to many Gram positive and a few Gram negative bacteria. Both the acid and the salt of endomycin have been extracted and these are stable to heat, acid, and alkali. Endomycin is soluble in alcohols but insoluble in most other organic solvents and in water. It is partially inactivated by horse serum and has a relatively low order of toxicity.

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MOSAIC OR BLACK STREAK DISEASE OF CYMBIDIUM ORCHIDS

D. D. JENSEN[1]

(Accepted for publication January 3, 1951)

Orchid plants have been cultivated intensively for many years, but very little research has been devoted to the virus diseases which infect them. So far as the writer is aware, the literature contains but three reports of experimental transmission of virus diseases from orchid plants. Nobrega (8) in 1947 transmitted a virus mechanically from *Dendrobium nobile* to *Nicotiana tabacum*, *N. rustica*, and *N. glutinosa* in Brazil. He considered the virus to belong to the cucumber-mosaic group. Attempts to infect healthy *Dendrobium nobile*, *Cattleya* sp., and *Oncidium* sp. by mechanical inoculation were unsuccessful. The aphid, *Macrosiphum luteum*, also failed to transmit the virus.

In 1949 the writer (4) reported transmission of Cattleya mosaic virus from diseased to healthy *Cattleya mossiae* and *C. trianaei* by means of the green-peach aphid, *Myzus persicae* (Sulzer). When this paper was published, mechanical transmission of Cattleya virus in expressed juice had not been accomplished and no hosts other than *Cattleya* had been demonstrated. In a later paper (5), mechanical transmission of this virus from *Cattleya* to *Cymbidium* was indicated and, in subsequent tests, has been confirmed.

Probably the earliest report in the literature in which an orchid disease was attributed to a virus was published in 1943 when Magee (6) described and illustrated some of the symptoms of a disease of Cymbidium in New South Wales which had been known as "black disease" but which he called orchid mosaic. Although Magee did not demonstrate the virus nature of Cymbidium mosaic, he expressed the opinion that the disease was caused by a virus and suggested that either spotted-wilt virus or lily-mosaic virus might be involved.

Bissett (1) briefly discussed the Cymbidium disease of Australia under the name black spot or mosaic. He believed the disease was due to bad potting mixtures and was not transferable from one plant to another.

In 1948 Moore (7) reported the same or a similar disease on Cymbidium at Kew and at St. Albans (Herts) in England. His preliminary tests indicated that neither tomato spotted-wilt virus nor cucumber-mosaic virus was present in the plants.

A mosaic disease of Cymbidiums is very prevalent in North America and probably throughout the world. Experiments at Berkeley, California,

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Grateful acknowledgment is made to the following companies who generously provided the experimental plants used in this investigation: Chase Gardens, Eugene, Oregon; Armacost & Royston, Inc., Los Angeles; E. W. McLellan Company of San Francisco; and Carbone Orchid Nursery, Berkeley, California.

reported by the writer (5) in abstract form, demonstrated the virus nature of Cymbidium mosaic. Gold (3) reported purification of the virus. In the electron microscope this preparation appeared to consist of elongate, sinuous rods approximately 18 mμ in diameter and 500 mμ in length. The disease studied in California appears to be the same as that occurring in England, and is probably the same as, or closely related to, that described from Australia by Magee. However, this is only a tentative conclusion based on the similarity of the symptoms and supported by the fact that orchid plants are shipped extensively throughout the world, thus making wide dissemination of the disease probable.

Of some interest is the fact that Cymbidium mosaic symptoms, indistinguishable from those produced experimentally during this study, have become one of the necessary hallmarks required of a hybrid, *Alexandri* var. *Westonbirt* F.C.C., which has proved to be exceptionally valuable in hybridization. *Cymbidium Alexandri* was produced in England in 1911 from the cross *C. eburneo-Lowianum* × *C. insigne*. The variety *Westonbirt* was an outstanding plant resulting from this cross. This plant apparently developed mosaic symptoms, because all known plants which have been derived from it by division or back bulbs have strong virus symptoms and growers have come to associate the symptoms with this variety. The presence of mosaic virus in a Cymbidium plant does not necessarily mean it is an *Alexandri Westonbirt*, for many hybrids have the disease; on the other hand, the absence of mosaic symptoms, in a plant purported to be derived from the original *Westonbirt* by vegetative propagation, would make the authenticity of the claim very doubtful.

TEST PLANTS

The high incidence of Cymbidium mosaic among commercial plants, particularly among those which have been involved in vegetative propagation over a period of years, makes older plants unsafe for use in virus transmission tests. The danger of unknowingly using plants already infected is increased by the fact that symptoms in some plants are not always well defined.

Although the possibility of virus transmission through the seed has not been entirely excluded, all available evidence indicates that seedlings are virus free.

The test plants used during this investigation were seedling Cymbidiums, all of which were free of the disease prior to experimental infection. Most of them were small seedlings grown in a greenhouse where insect control is maintained. These varied in size from plants of a single bulb to plants with one bulb and one or two new shoots 1 to 10 in. long. In addition to the young seedlings used, some of the experimental plants were *Cairngarn* and *Margaret* seedlings which had grown in the ground for 4 years and were large plants. Each of these was divided into two or three sister plants. For each plant infected a sister plant was kept as a control

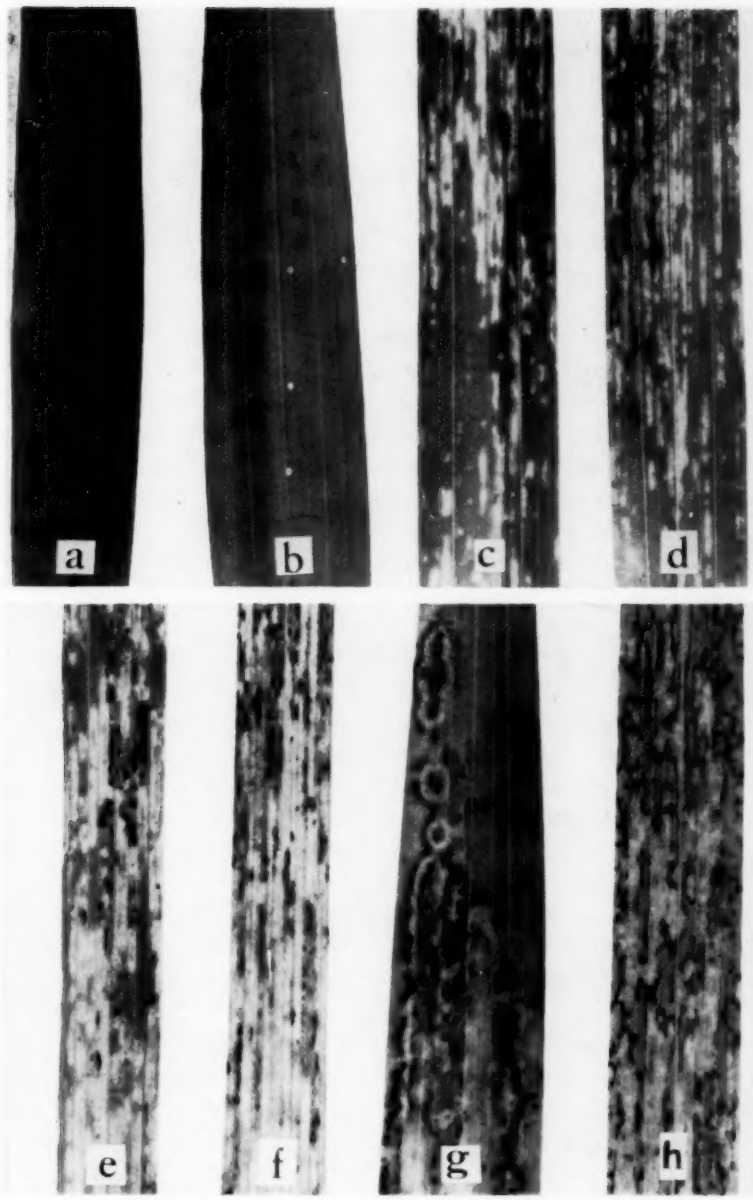

FIG. 1. Healthy and mosaic-diseased Cymbidium leaves: upper and lower surfaces of (a, b) healthy leaves, and (e, d) diseased leaves showing mottle without necrosis; upper (e, g) and lower (f, h) surfaces of leaves with mosaic mottle, necrotic streaks and rings.

(Fig. 1, a, b) to demonstrate the original noninfectivity of the test plant used.

All of the young seedlings have been grown in the greenhouse during the present experiments. The large seedlings, grown in 7- to 10-in. pots,

have been kept in the greenhouse part of the time and in outside screen cages part of the time. After inoculation, all test plants were held in the greenhouse until after definite symptoms had developed. All control plants have remained free of mosaic.

TRANSMISSION OF CYMBIDIUM VIRUS

Cymbidium mosaic virus was readily transmissible to healthy seedlings when leaves of the test plants were rubbed with diseased juice in the presence of carborundum powder. Of 29 test plants inoculated by this method, 26 became infected; the three which remained healthy were inoculated with juice in which the virus apparently had been inactivated by heat treatment.

Most of the test plants were inoculated on the leaves of a young shoot and also on the leaves of the back bulb. Transmission was also accomplished when only the leaves of an older back bulb were inoculated on a large plant. However, the development of symptoms in this plant was delayed in comparison with similar plants inoculated on the new shoots as well as on the older leaves.

Experiments to determine possible insect vectors of Cymbidium mosaic virus are in progress, but the results are as yet inconclusive.

SYMPTOMS

The symptoms of Cymbidium mosaic disease vary considerably in pattern and severity. Typically, no evidence of the disease develops at the site of inoculation. The virus becomes systemic and symptoms appear first on the new growth. The amount of growth made by a given leaf after infection determines the nature and extent of the symptoms on that leaf. Typically, mottle does not develop on leaf tissue already produced prior to or immediately after infection. The symptoms appear first on the basal portion of the leaves and the amount of apical leaf tissue without symptoms is a measure of the growth prior to infection. The symptoms develop first on the new shoots near the base of the leaf making the most rapid growth. This may be any leaf from the youngest to one of the older leaves, depending upon the maturity of the shoot.

Symptom development on young seedlings follows a rather consistent sequence. The test plants used had a single bulb and a new shoot with four to seven expanding leaves, the longest of which ranged from 5 to 10 in. On plants making good growth, the first evidence of the disease appears approximately 6 weeks after infection and consists of small, inconspicuous, elongate, chlorotic areas. Frequently these are initially confined to the leaf blade on one side of the midrib. After a few days the spots and streaks become more sharply defined. Within 1 to 3 weeks after symptoms first appear, the affected area enlarges as a pale, chlorotic patch approximately $\frac{3}{4}$ in. long, broken only rarely by a few small streaks of darker green tissue. The new growth subsequently produced below the site of the first symptoms develops irregular, elongate streaks of chlorotic tissue scattered throughout

the normal darker green tissue; the contrast between the light and dark green areas gradually becomes more marked, and within 3 or 4 months after infection sufficient symptom-bearing tissue has grown to make the disease conspicuous.

Within 6 months after infection, black spots and streaks sometimes appear on the under side of the older leaves of small seedlings. Necrosis in the youngest leaves is uncommon and usually appears only in very severe cases. On some plants brown spots and streaks develop on the under side of the older leaves that matured before infection. They appear several months after typical mottle develops in new growth.

Symptom development on large plants making rapid growth is frequently faster than on small seedlings with late-stage mottle symptoms (Fig. 1, c, d), attaining good definition several weeks earlier than is true of small seedlings. A new shoot 4 in. long when inoculated will attain a length of about 13 in. before symptoms are definite. In these experiments this required from 5 to 6 weeks. Within 3 to 6 months after infection, some large plants develop brown to black spots, streaks, and rings on the older diseased leaves. These appear first on the under side but in severe cases necrosis extends through to the upper surface. The necrotic patterns (Fig. 1, e–h) produced on the leaves of large plants are more diverse than occur on small seedlings inoculated from the same virus source. There is also more variability of mottle symptoms in older plants than in young plants.

Although the factors which determine the development of necrotic areas on the leaves are not yet understood, environmental conditions, such as high temperatures, are probably involved. During September 1949 two large plants, one infected 3 months and the other 7 months earlier, developed severe necrosis simultaneously (Fig. 2, a–d). The plant infected for 7 months had had mild black spotting for more than 2 months previously, but the plant infected for 3 months had no necrosis 2 weeks prior to development of severe symptoms. Both had been held in the greenhouse, where temperatures had been higher during September than during earlier months. Plants growing in outside screen cages also developed necrotic symptoms but of a milder type than those under glass. A thick liquid exudate was produced from the severely necrotic tissue. Leaves having extensive and severe necrotic areas are dropped prematurely.

Plants with necrotic spots and streaks on the older leaves usually produce new shoots bearing strong mottle symptoms. Necrosis usually is not extensive in these new shoots until the leaves reach or approach maximum size, but small black spots and streaks may appear while the shoots are still only a few inches long. Some plants have conspicuous and extensive mottle symptoms but no necrosis.

The effect of the disease on the general growth and vigor of the plant is variable. Some plants have definite but relatively mild symptoms; on others the amount of chlorotic and necrotic tissue is so great that the growth is severely retarded. Many of these plants are destroyed by the

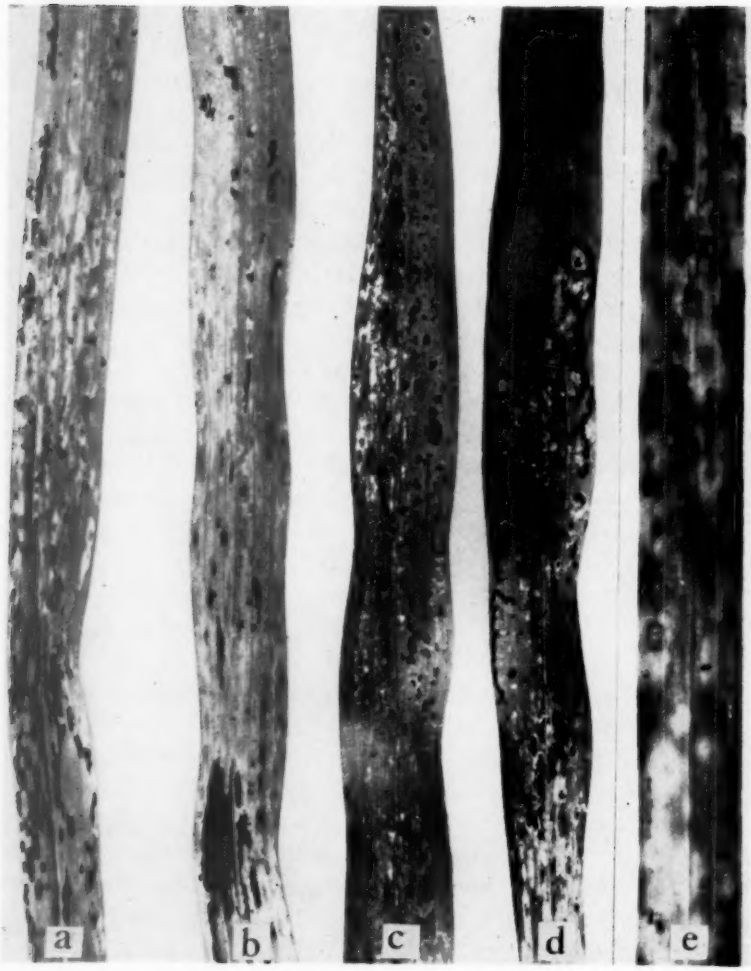

FIG. 2. Leaves from an experimentally infected Cymbidium plant, with mottle, severe necrosis, and malformation 7 months after infection: (a, c) upper surface of leaves; (b, d) lower surface; (e) leaf with atypical symptoms from naturally infected plant.

growers. There is no evidence that mosaic plants recover, but the mottling, which is so evident on the young leaves, sometimes becomes inconspicuous by the time the leaves are old. It is also probable that the mottle symptom is more strongly expressed in the same plant during some seasons than during others.

There is no evidence that Cymbidium mosaic virus causes breaking in the color of flowers or malformation of the flowers. However, flower production is affected indirectly as a result of growth retardation.

INCUBATION PERIOD OF SYMPTOMS

The time elapsing between inoculation and symptom expression on plants growing well at time of infection ranged from 37 to 72 days and

averaged 43 days (Table 1). Although the tests were too few to permit definite conclusions, the plants inoculated with juice which had aged *in vitro* for 7 days or which had been heated at 60° and 65° C. for 10 min. had a relatively long incubation period, despite the fact that the test plants had grown at a normal rate for several weeks after inoculation.

HEAT TREATMENT OF DISEASED PLANTS

A preliminary experiment was conducted to determine whether Cymbidium mosaic virus could be inactivated in living plants. Five diseased plants were used. Two had bare roots, having grown in vermiculite, two were growing in soil, and one was potted in osmunda. The plants were completely submerged for 2 hr. in water held at 45° C. To insure uniform temperature throughout the potting medium, the pots were lifted out of the water several times early in the experiment so that the water could drain out and be replaced by hot water. After this had been repeated several times, a thermometer was inserted in the soil, where it registered 45° C. The porosity of the osmunda permitted very rapid exchange of water when this pot was lifted up and down in the water.

The immediate effects of the hot water on the plants were not severe, the only evident injury being a burning of the older leaves after a few days. For 3 weeks all plants were thought to have survived the treatment successfully, but during the second three weeks four of the plants died, the one in osmunda being the only survivor. This plant produced two new shoots within 2 months after the treatment and both shoots developed typical mosaic symptoms. Although few plants were used in this test, the results suggest that Cymbidium mosaic probably cannot be controlled by immersing diseased plants in hot water.

EVIDENCE THAT CYMBIDIUM-MOSAIC VIRUS IS NOT TRANSMITTED THROUGH SEED

The great majority of Cymbidiums grown commercially are hybrids. Although there are between 30 and 40 species described, only six or seven have been used commonly in hybridization. Boyle (2) lists 1,343 Cymbidium hybrids, most of which have been produced in the last 20 to 30 years. The majority of these were produced by using other hybrids, rather than species, as parents.

Cymbidiums have been cultivated on a commercial scale in the United States for a relatively short time, most of the American interest in this orchid having developed in the last 15 to 20 years. Nearly all the flowering Cymbidiums grown commercially in the United States at the present time are hybrids developed in England, where they have been grown and hybridized for a much longer time. Most of these were acquired as seedlings, as plant divisions, or as back bulbs. Some seeds were shipped from England and germinated in America.

During recent years growers in America have been hybridizing Cymbidiums extensively and hundreds of thousands of small seedlings are in vari-

TABLE 1.—*Incubation period of Cymbidium mosaic virus in Cymbidium seedlings. Mechanical inoculation with fresh juice, heat-treated juice, and juice aged in vitro at room temperature*[a]

Treatment of inoculum juice before inoculation	Number of plants inoculated	Number of plants infected	Range of incubation period, in days
Thermal inactivation tests (10 min. at each temperature)			
45° C.	3	3	43–250[b]
50	3	3	43– 50
55	3	3	41– 66
60	2	2	68– 72
65	2	1	215[b]
70	2	0	
Aging *in vitro* at room temperature			
48 hr.	1	1	41
68 hr.	1	1	45
7 days	1	1	65
Fresh, untreated juice	11	11	37–53

[a] Thirty untreated control plants remained healthy.

[b] The plant which required 250 days to develop symptoms consisted of a single bulb bearing old leaves at the time of inoculation. No new shoot growth occurred during the first 8 months after infection. The plant requiring 215 days consisted of a single shoot which developed a bulb several weeks after inoculation resulting in a retardation of leaf growth.

ous stages of growth. The high incidence of mosaic disease among commercial Cymbidium hybrids suggests the possibility of seed transmission of the virus. A survey was made in California to determine the prevalence of mosaic and to obtain evidence as to the likelihood of virus passage through the seed. Special attention was given to hybrids germinated in California and whose history is known. Most of the hybrids produced in America are still relatively young seedlings, 3 years old or less, but several thousand older plants exist which were grown from seed in California. During 1950 approximately 17,000 of these larger Cymbidium hybrids 3 to 9 years of age and 50,000 1- to 3-year-old seedling hybrids were inspected (Table 2). All

TABLE 2.—*Incidence of mosaic disease in Cymbidium hybrids grown from seed in California, involving several hundred parent combinations, many of which were infected with mosaic virus*

Age (in years since germination)	Number of plants inspected	Years grown in lath house or in open ground	Percentage diseased
1– 3	40,000–50,000	0	0
3– 4	4,000	0	0
4	10,500	1–2	0
5	500	2	0
6– 7	1,600	3–4	0
8– 9	350	4–5	0
20–25	300[a]	10	14
20–25	350[a]	10	5

[a] The plants listed are not the original individual seedlings, but were grown from back bulbs and divisions made throughout the years.

were free of mosaic symptoms. Although the incidence of disease among the parents of these seedlings is not known, several hundred parent combinations were used including many which were infected with mosaic. These facts indicate that (a) the virus seldom if ever goes through the seed to infect the seedlings, or (b) if the virus is seed-transmitted to some extent, the growth of the young seedlings may be affected by the virus in such a deleterious manner that infected plants are culled out and discarded at the time the plants are transferred from the germination flasks to community pots.

Cymbidium mosaic was found in plants of only three hybrids grown from seed in California (Table 2). Two of these hybrids were produced in California and the third was grown from English seed; all three were produced approximately 20 years ago. It is probable that the seedlings were originally virus-free, but that some of them acquired the disease by natural spread. Increasing the number of plants by division and by growing plants from back bulbs has multiplied the number of diseased plants of the respective hybrids. Records are not available to permit determination of how many individual plants originally became infected by natural spread.

RATE OF DISEASE SPREAD

There is little direct evidence available bearing on the rate at which Cymbidium mosaic spreads among previously healthy plants. According to table 2, approximately 13,000 individual seedlings have grown in lath houses or in the open ground for 1 to 5 years without becoming infected, despite the fact that diseased plants were growing in relatively close proximity the entire time. However, in the same localities, hybrids produced 20 to 25 years ago had acquired the disease. It is thus apparent that the rate of natural spread in California may be very slow in some situations.

It also seems evident, however, that mosaic virus is sometimes readily transmitted to healthy plants in nature. A survey of the older commercial Cymbidium hybrids in California revealed a very high incidence of the disease, infection ranging from 15 to 70 per cent with an over-all mean of approximately 40 per cent of the plants inspected. The incidence of disease is high even among many of the plants derived from young hybrid seedlings imported 15 years ago from England, where the disease is known to occur. It is not known whether the young hybrids shipped from England years ago were infected before shipment or acquired the disease after arrival in the United States. It seems probable that at times considerable natural spread has occurred in the United States as well as in England, if it can be assumed that the virus is not transmitted through the seed.

HOST RANGE

No evidence can be found that any Cymbidium species or hybrids are immune from the virus. Among the species available for inspection, symptoms were found in at least one *C. Lowianum* and in approximately half

of 200 *C. insigne* plants growing in a lath house. The list of commercial varieties found to be diseased would be very long and it is probable that mosaic could be found in some plants of most of the hybrids which have become widely distributed during the last 20 to 30 years.

In an attempt to find other hosts of Cymbidium mosaic virus, 24 species of plants representing 20 genera in 15 families of plants were mechanically inoculated. No infection was obtained in any plant other than Cymbidium. Attempts to transmit the virus by means of aphids to *Cattleya* orchids also failed. The plants tested are: sugar beet (*Beta vulgaris* L.); China aster (*Callistephus chinensis* Nees); pak choi (*Brassica chinensis* L.); radish (*Raphanus sativus* L.) var. White Icicle; cauliflower (*Brassica oleraceae* var. *botrytis*); squash (*Cucurbita maxima* Duschesne); white-stem filaree (*Erodium moschatum* L'Her.); corn (*Zea mays* L.) var. Golden Bantam; *Freesia refracta* Klatt.; bean (*Phaseolus vulgaris* L.) var. Blue Lake; soybean (*Soya max* Piper); crimson clover (*Trifolium incarnatum* L.); red clover (*Trifolium procumbens* L.); *Cattleya triànaei* L. & R.; plantain (*Plantago major* L.); cyclamen (*Cyclamen indicum* L.); primrose (*Primula obconica* Hance); bracted strawberry (*Fragaria bracteata* Heller); loganberry (*Rubus loganobaccus* Bailey); tomato (*Lycopersicon esculentum* Mill.) var. Earliana; tobacco (*Nicotiana tabacum* L.) var. Turkish; tobacco (*N. glutinosa* L.); garden nasturtium (*Tropaeolum majus* L.); celery (*Apium graveolens* L.) var. Golden Self Blanching; violet (*Viola* sp.).

IDENTITY OF CYMBIDIUM-MOSAIC VIRUS

The several distinct symptom types occurring in Cymbidium suggest that this genus may be infected by more than one virus or virus strain, even though the great majority of diseased plants have symptoms typical of those described in this paper. Although the identity of the virus or viruses involved is not known, experimental results strongly indicate that the virus studied in this investigation is not one of the commonly known viruses. This does not imply that Cymbidium may not be a host, with or without symptoms of one or more known viruses.

The high percentage of Cymbidium plants experimentally infected with Cymbidium-mosaic virus by juice inoculation, even after aging *in vitro* for 7 days and after heat treatment at 65° C. for 10 min., demonstrates that the virus is very stable and should be readily transmissible to susceptible plant species. Failure of the virus to cause disease in such plants as tobacco, tomato, squash, and some legumes and crucifers makes it, therefore, highly improbable that this virus belongs among the large group of viruses which infect one or more of these hosts.

Magee suggested that Cymbidium mosaic in Australia might be caused by spotted-wilt virus. Although in the writer's experience garden nasturtium, *Tropaeolum majus*, is an excellent host of the spotted-wilt virus, the amount of transmission from nasturtium to nasturtium being nearly 100

per cent even after the source plant has been infected for a few months, repeated attempts to transmit Cymbidium-mosaic virus to nasturtium have failed. In two tests, spotted-wilt virus from Calla lily also failed to infect Cymbidium although it produced severe symptoms in nasturtium inoculated at the same time. The fact that spotted-wilt virus is so readily inactivated *in vitro* and by heat is further evidence that typical Cymbidium mosaic is not caused by this virus.

OTHER SYMPTOMS ON CYMBIDIUM

Tip burn or dieback (Fig. 3, d) is common on many Cymbidiums in California, appearing primarily on the mature and older leaves. Usually the effect is confined to the apical 2 to 8 in. of the leaf. Although, to the writer's knowledge, no experiments dealing with this problem have been reported in the literature, the opinion is prevalent among growers that the symptom is nutritional in origin. Some of the plants used by the writer had tip burn when transplanted from the ground in a lathhouse to fresh potting material in the greenhouse. During the first year after the transfer only a slight amount of tip burn developed on the maturing leaves. This suggests that the cause of the disorder had been largely corrected, temporarily at least, by the new potting material and the greenhouse environment.

Occasional Cymbidium plants have leaf symptoms which suggest virus infection but which are quite distinct in pattern from those commonly encountered. The symptoms are not confined to a single leaf, but appear with considerable consistency on the various shoots of the plants.

In a limited number of tests involving several different disease symptoms, transmission has thus far been accomplished only with the virus producing the disease illustrated in figure 2, e. On the source plant the symptoms consisted of a conspicuous yellow and green leaf mottle with some of the yellow areas enclosing islands of green tissue. Virus was transmitted from this plant by juice inoculation to the single small Cymbidium seedling tested. The first symptoms appeared 51 days after inoculation and at 4 months they were indistinguishable from the symptoms produced by typical Cymbidium mosaic virus. The reason the symptoms on this source plant were aberrant is not known. Possible explanations include (a) a difference in host response; (b) a virus complex, only one component of which may have been transmitted in the test conducted; and (c) a distinct virus strain.

Several plants have symptoms characterized by a yellow mottle enclosing a green area. Some of these areas are diamond-shaped and the symptom is designated as diamond mottle (Fig. 3, a). This symptom is most distinct on the younger leaves, often changing to a light green streak as the leaves mature. There is evidence that symptom production by this virus may be very slow. A single *Margaret* seedling, approximately 6 years old, was divided into two plants July 27, 1949. During the 6 months prior

to division the plant had been under observation and had no virus symptoms. Three months after division one of the sister plants developed diamond mottle in two new shoots. By May 1950 the symptom had changed

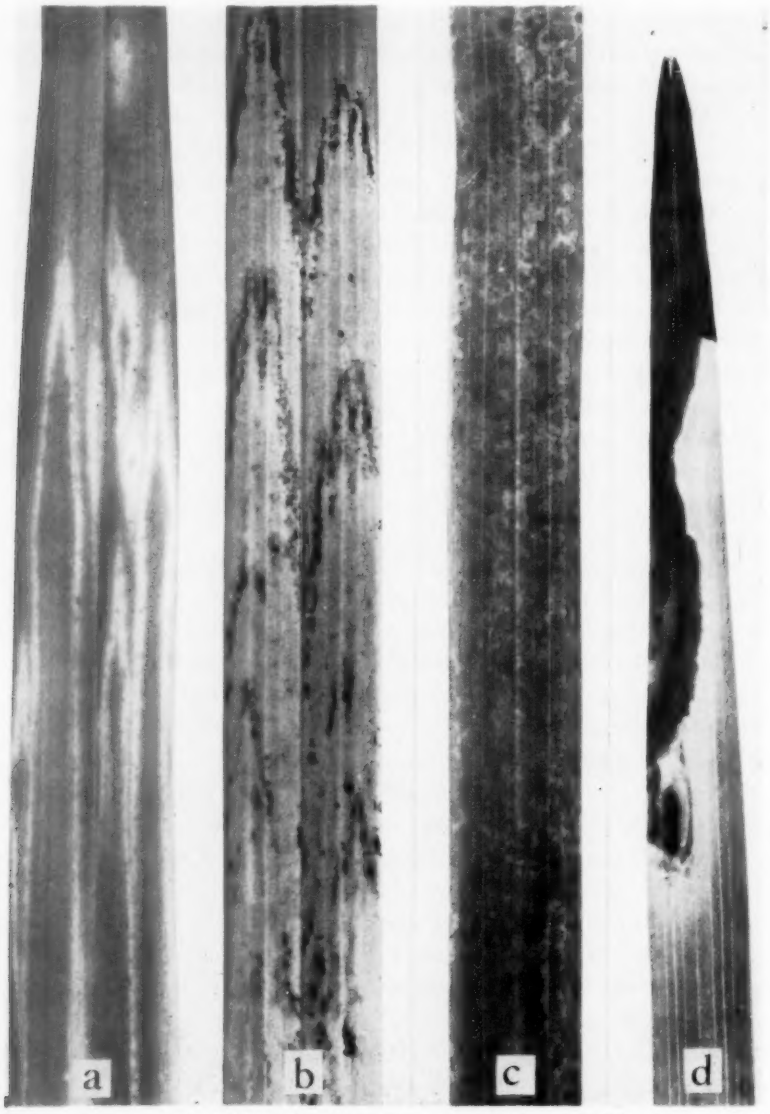

FIG. 3. (a, b, c) Cymbidium leaf symptoms, probably caused by virus, which have not been produced experimentally. (c) Cymbidium leaf with tip burn or dieback from unknown cause.

to a light green streak down the middle of the leaves. In addition, circular chlorotic areas developed near the distal portion of some of the older leaves. No abnormal symptoms developed in the sister plant until May 1950 when similar yellow areas appeared in the subapical portion of some of the older leaves. In June 1950 the new growth developed the characteristic dia-

mond-mottle symptom on the young shoots. Since both sister plants developed the same symptoms whereas 24 other seedlings of the same age and source remained healthy, it appears probable that the plant became infected prior to division even though one of the two plants did not develop symptoms until almost a year after division.

Several plants have been found which have leaf symptoms characterized by brown to black flecks and streaks which coalesce to form patterns typified by the leaf illustrated in figure 3, b. The tissue covered by the dark areas usually is somewhat sunken. This symptom has been found both in the presence and absence of leaf mottle.

Another symptom probably caused by virus is illustrated in figure 3, c. The normal green color of the leaves is broken by light green tissue which usually takes the form of small chlorotic rings or fragments of rings, many of which coalesce. Symptoms illustrated in figure 3 have not yet been produced experimentally.

SUMMARY

Mosaic or black streak, prevalent disease of Cymbidium orchids, is described and shown to be caused by a virus. It occurs throughout North America and England, and is probably the same disease which has been reported from Australia. Three species of *Cymbidium* and many hybrids have been found naturally infected with the virus.

The first symptoms develop in approximately 6 weeks after infection: small, faintly chlorotic streaks and patches on the younger leaves. Subsequently the mosaic mottle becomes conspicuous and is followed by necrosis in the form of spots, streaks, and rings. No symptoms have been observed in the flowers. Considerable variation occurs in the severity of the disease, some plants being badly damaged while others are only slightly affected.

The virus is readily transmissible from *Cymbidium* to *Cymbidium* by means of juice inoculation. It is a stable virus, retaining infectivity *in vitro* for at least 7 days at room temperature. It also remains infective in juice heated for 10 min. at 65°, but is inactivated at 70° C. The virus is not inactivated in plants submerged for 2 hr. in water held at 45° C.

The available evidence indicates that seed transmission of Cymbidium-mosaic virus is unlikely.

The host range of Cymbidium-mosaic virus appears to be limited, since no transmission was accomplished when the virus was inoculated mechanically into 24 species of plants representing 20 genera and 15 families. The identity of the virus is unknown. Its failure to infect other plant groups suggests that it may be a previously undescribed virus.

In addition to mosaic, Cymbidium disease symptoms are described which may prove to be due to one or more viruses distinct from that which causes the prevalent mosaic.

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A TOMATO DISEASE RESEMBLING CURLY TOP

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During October, 1944, Dr. A. L. Harrison of Bradenton, Florida, wrote to N. J. Giddings concerning the occurrence of a tomato disease very similar in appearance to curly top. This disease, reported to have been present for several years, does not affect the spring crop but is found in the fall crop. The amount of infection occurring in a field was only a trace to about 3 per cent, but many affected plants died.

Some cuttings were sent to the Sugar Plant Investigations field station at Riverside, California, and the symptoms on them were promptly identified by staff members as curly top (Fig. 1). Efforts to recover curly-top virus from these specimens failed, and the cuttings did not survive for very long.

In October, 1945, Harrison reported that the disease was again present in fields around Bradenton but that there was not more than one per cent in any of the fields where observations had been made. Several diseased plants were sent to Riverside and the symptoms on these were indistinguishable from curly top (Fig. 1). These plants survived much better than the cuttings and numerous attempts to recover curly-top virus from them failed.

Transmission experiments both at Bradenton and at Riverside have shown that this disease is not transmitted by juice-inoculation methods, but is readily transmitted by grafting. The disease has been maintained by the grafting method. Some efforts have been made to obtain dodder transmission, without success, although something suggestive of very mild symptoms appeared in two of the tomato plants to which it was hoped to transmit the disease.

In the greenhouse, the tomato plants that had been infected by graft inoculation developed very mild symptoms and it was sometimes difficult to decide that they were infected. Occasionally the symptoms became more evident and, very rarely, a plant developed such severe symptoms that death seemed probable, but it soon recovered and new growth had only slight symptoms.

Experiments involving the tomato varieties Pearson, Golden Ponderosa, and Jubilee have indicated that Jubilee was a little more susceptible than the other two. Slight symptoms were induced on plants of *Datura stramonium* L. and of *Lycopersicon hirsutum* Humb. & Bonpl., that had been grafted with scions from diseased tomato, and there was some question as to whether any true symptoms were induced on plants of *Nicotiana glutinosa*

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L. The most conspicuous symptom on any of these plants was mild vein-clearing. Similar grafts induced no symptoms on plants of *Lycopersicon pimpinellifolium* (Justl.) Mill., *L. peruvianum* (L.) Mill., *Nicotiana tabacum* L., *N. acuminata* Hook., *N. glauca* Graham, *Datura meteloides* DC., *Phytolacca americana* L., and *Ricinus communis* L.

It has been suggested that this disease might be the result of infection by the aster-yellows virus, but our evidence to date would indicate that it is different from yellows-infected tomatoes as described by Kunkel.[2]

There are, of course, different strains of the aster-yellows virus and it is

FIG. 1. Tomato plants with severe symptoms of (left) curly top and (right) a disease resembling curly top.

possible that one of the strains of this virus is responsible for the disease. We know also that there are many strains of the curly-top virus present in the United States and that viruses that appear to be varieties or strains of the curly-top virus complex, and which are transmitted by leafhoppers other than *Circulifer tenellus*, occur in Argentina[3] and Brazil.[4] The known characteristics of the disease under consideration more closely resemble

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those of curly top than those of aster yellows. However, whether the causal virus is related to the curly-top virus complex probably cannot be determined definitely until it is possible to make studies by methods of transmission other than tissue union.

During 1947, a number of diseased tomato plants were received from Ysleta, Weslaco, and Jacksonville, Texas. These plants had symptoms similar to those induced by the above described disease and had been suspected of being that disease, but curly-top virus, *Ruga verrucosans* Carsner & Bennett, was recovered from all shipments.

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HISTOLOGICAL STUDIES OF A TOMATO DISEASE RESEMBLING CURLY TOP

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(Accepted for publication December 15, 1950)

Histological studies were made of tomato plants infected by a disease that induced symptoms resembling curly top.[1]

These studies show differences between this disease and curly top that are undetectable from the external anatomy of the infected plants.

Sectioned material was stained with iron-haematoxylin and counterstained with safranin and light green in clove oil. A transverse section through the vascular ring of a healthy tomato leaf stem is shown in A of figure 1. The internal and external phloem cells are in small evenly spaced groups. Figure 1, B, shows the vascular tissues of a similar tomato stem infected by one of the virulent strains of curly-top virus. There is necrosis and degeneration of the phloem tissue causing disorganization and distortion in the vascular ring. Figure 1, C is of comparable tissue infected with this new tomato disease. There is abnormal production of phloem tissue at irregular intervals and great disorganization of the vascular ring, but no necrosis or degeneration as in severely diseased curly-top plants.

Figure 1, D, E, and F show some of the effects of curly top and the new tomato disease in greater detail as compared with healthy tissue. Portions of the vascular rings of tomato stems are shown. Figure 1, D shows healthy tissue; figure 1, E, curly-top-infected tissue with phloem necrosis and degeneration; F shows the effect of the new disease. In this case, in addition to proliferation of the phloem cells, there is pronounced and characteristic hypertrophy of the phloem parenchyma.

The vein distortion of the leaves of tomato plants infected with curly top and this new disease are caused by abnormal stimulation in growth of parenchyma cells as well as hypertrophy of this tissue. In addition, curly top causes necrosis of phloem tissue in the leaves, whereas this latter disease does not.

These studies were made on tomato plants infected in the greenhouse. It is not readily apparent from these investigations just how the disease causes severe injury to plants in the field.

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[1] Giddings, N. J., C. W. Bennett, and A. L. Harrison. A tomato disease resembling curly top. Phytopath. 41: 415–417. 1951.

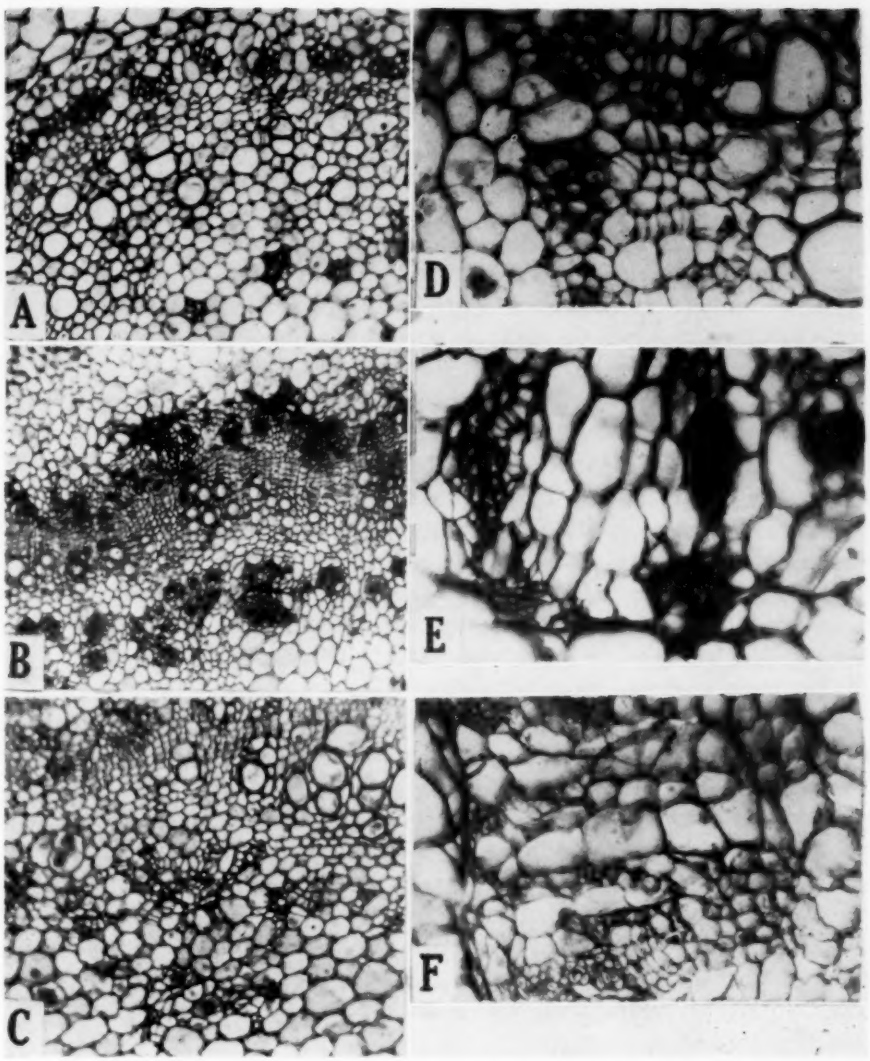

FIG. 1. Transverse sections of vascular rings of tomato leaf stems and side branches: (A) healthy leaf stem, (B) stem infected with curly top, and (C) stem infected with tomato disease resembling curly top (approximately 58×); (D) healthy side branch, (E) side branch infected with curly top, and (F) side branch infected with tomato disease resembling curly top (approx. 216×).

THE PRODUCTION AND ROLE OF ANTIBIOTICS IN THE SOIL: I. THE FATE OF STREPTOMYCIN

PAUL SIMINOFF AND DAVID GOTTLIEB[1]

(Accepted for publication January 10, 1951)

The questions whether antibiotic substances are produced in the soil and whether such materials exert any influence on its microbiological population are pertinent to the control of soil-borne diseases. An answer to these questions would contribute to an understanding of the dynamics of biological activity in the soil and would indicate whether control of soil-borne plant pathogens by the use of antagonistic organisms could be attributed to the production of antibiotics. Until now, the evidence has been primarily indirect, and little attention has been focused on the role of the soil in modifying any antibiotic effects that may occur.

The soil harbors a highly complex and extensively distributed microbial population of which many members demonstrate antagonistic effects on laboratory media (16). A number of investigators have shown that when nonindigenous bacteria are inoculated into fresh soil, they rapidly die out; yet these same organisms are able to survive in sterile soil (8, 15, 18). As early as 1910, Greig-Smith (5) postulated the existence of bacteriotoxins in the soil, but this hypothesis was disputed by Hutchinson and Thaysen (6). Later, Lewis (9) made a careful study of the antibiotic principle elaborated by *Pseudomonas fluorescens*. The principle could be adsorbed from culture media by charcoal and field soil. He cultured the antagonist in sterile manured soil and extracted the soil with water, but could find no evidence of the antibiotic in the extracts. Waksman and Woodruff (19) obtained ether extracts of two soils which demonstrated actinomycinlike activity against *Bacillus subtilis* and *Sarcina lutea*. When actinomycin was added to soil, it was largely inactivated and these investigators attributed this effect to its adsorption by the soil or destruction by soil microorganisms, concluding that microbial toxins of the actinomycin type exert very little effect on the soil population.

Some investigators have regarded the production of antibiotics as a competitive mechanism which enables a species to survive in the soil. Singh (14) noted that antagonistic *Myxobacteria* produced fruiting bodies on laboratory media only when lysis-susceptible bacteria were present. Schiller (13) developed a method of "forced antagonism" by feeding a culture of one organism to another, forcing the second to develop the capacity to destroy the first. Dubos' method of soil enrichment (3) is, in essence, an application of Schiller's technique. The theoretical basis

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The authors wish to acknowledge the helpful suggestions of Dr. O. H. Sears and Dr. John Gieseking during the course of this research.

for the soil-enrichment method has been criticized by Waksman and Schatz (20), who point out that there is insufficient proof that such antagonistic effects are due to antibiosis, and that the production of an antibiotic may merely be an accidental characteristic.

Certain correlations have been obtained between the production of a toxic principle by an antagonist and its ability to limit the development of a susceptible organism in nature. Nickell and Burkholder (11) demonstrated that an antagonistic actinomycete which inhibited the growth of *Azotobacter vinelandii* on laboratory media also prevented the development of the bacterium in amended soil cultures. They were also able to obtain an extract of a soil culture of the actinomycete which demonstrated marked antibacterial activity. Sanford and Broadfoot (12) found that both antagonistic soil organisms themselves and their filtrates controlled the pathogenicity of *Ophiobolus graminis* for wheat seedlings grown in sterilized soil. Weindling (21) and Weindling and Fawcett (22) showed that when *Trichoderma lignorum* was added to sterilized soil simultaneously with the pathogen *Rhizoctonia solani*, no damping-off of citrus seedlings occurred. They attributed control to a thermolabile, alkali-unstable toxic principle elaborated by the antagonist. Numerous other instances of this type of biological control of plant disease have been reported (1, 2, 7), but investigations on the mechanisms of such antagonism are lacking.

In any investigation concerning the production of antibiotic substances in the soil and their effect on susceptible organisms growing in the same environment, general competitive effects must be clearly distinguished from what we now term antibiosis. Therefore, this preliminary investigation includes 1) biological studies, in which an antagonist which produces a known, chemically defined antibiotic was cultured with a susceptible organism in the soil and the population changes of each were measured, and 2) a physico-chemical study of the effect of the soil and other natural substrates upon the antibiotic in question.

BIOLOGICAL STUDY: METHODS AND MATERIALS

The two organisms selected for study were the indigenous soil forms *Streptomyces griseus* and *Bacillus subtilis*. *S. griseus* produces the chemically defined antibiotic, streptomycin, to which *B. subtilis* is strongly sensitive. The actinomycete was grown in the soil cultures before infestation with the bacterium in order to permit its unhampered development and possible production of the antibiotic in soil. A dark, heavy prairie soil was coarse-sieved and 20-gm. portions were placed in 125-ml. Erlenmeyer flasks. One-half of the flasks were amended with 0.5 gm. of Bacto-tryptone. All flasks were plugged with cotton and autoclaved for 1 hr. at 15 lb. pressure. The treated and untreated soil cultures were then further divided into three series: 1) control flasks to contain *Bacillus*

subtilis only; 2) control flasks to contain *Streptomyces griseus* only; and 3) flasks to contain *B. subtilis* and *S. griseus* together.

A culture of a streptomycin-producing strain of *S. griseus* was first grown on Emerson's agar slants and tested for production of the antibiotic. This organism was then transferred to Emerson's agar in 2-qt. milk bottles for preparation of a large-scale inoculum. When spores had developed (usually after 4 days' incubation at 28° C.), they were washed or scraped off with the aid of a sterile 1 : 1000 solution of Vatsol. The spores of *S. griseus* were then filtered through two layers of sterile Johnson & Johnson "Rapid Flo" filter discs to remove adhering mycelia and agar, and the suspension was brought to a final volume of 100 ml. with sterile distilled water. One ml. of this spore suspension was pipetted into each of the flasks of series 2 and series 3. The soils were brought to 60 per cent of moisture-holding capacity with sterile distilled water and incubated at 28° C. Thereafter, water was added every 2 weeks to maintain the proper moisture level. At the end of 17 days, duplicate cultures were extracted with 20 ml. of sterile distilled water on a reciprocal shaker for 2 hr. and then plated out in triplicate dilutions from 1 : 100 to 1 : 10,000,000. Counts were made of those plates containing 30 to 300 colonies and the number of viable cells was computed on the basis of the air-dry weight of soil. The pH of the extracted soils was also determined. The flasks in series 1 and series 3 were then inoculated with 0.1 ml. of a washed 24-hr. suspension of *B. subtilis* and the soil cultures were shaken to distribute the inoculum as evenly as possible. At intervals of 16, 31, and 45 days after infestation of the soil cultures with the susceptible bacterium, counts were made of the antagonist and the bacterium, and the pH of duplicate amended and unamended cultures in each of the series was measured.

BIOLOGICAL STUDY: RESULTS

The presence of *S. griseus* in soil inhibited the multiplication of *B. subtilis* (Table 1). The population of the bacterium when grown in the sterile unamended soil increased from 200 viable cells to 25 million per gm. of soil in 45 days. Under the same conditions, *S. griseus* increased from 16 million to 268 million viable cells. However, when *B. subtilis* was added to the soil which already contained the antagonist, the bacterium at first increased, though to less than one-half the population of the control, and thereafter decreased until no viable cells could be detected. During this period, the growth of the actinomycete in the mixed culture increased approximately the same extent as its control. It is notable that even when allowed to grow in the absence of the antagonist, *B. subtilis* did not multiply at a rate comparable to that of *S. griseus*.

A similar inhibitory effect by *S. griseus* on *B. subtilis* occurred in the presence of tryptone; the bacterium tended to die out. The maximum growth phase of the actinomycete was rapidly reached before the susceptible bacterium was added and, thereafter, the population of the

actinomycete declined. In the mixed cultures, *B. subtilis* could not be detected until the end of the 45-day period when 6.2 million cells were counted, at which time the population of *S. griseus* had reached its lowest point. It is interesting that in both the amended and unamended soils, an actinomycete population of about 80 million cells per gm. of soil permitted a population of *B. subtilis* of 4 to 6 million viable cells. When the number of bacteria and actinomycetes in the controls containing the tryptone are compared with the numbers in the unamended controls, the stimulatory effect of the tryptone is apparent.

TABLE 1.—*The growth of Bacillus subtilis in soil cultures in the presence of a streptomycin-producing strain of Streptomyces griseus*

Treatment	Culture	Incubation period[a] (days)	Number of viable cells, millions per gm. of soil		pH
			B. subtilis	*S. griseus*	
Unamended soil	*B. subtilis* control	0	20×10^{-5}	.....	6.20
		16	11.5	.....	6.20
		31	14.5	.....	6.10
		45	25.4	.....	5.92
	S. griseus control	0	.....	16.0	5.98
		16	.....	122.5	6.25
		31	.....	294.0	7.05
		45	.....	268.0	7.09
	B. subtilis + *S. griseus*	0	20×10^{-5}	16.0	5.98
		16	4.5	84.0	6.24
		31	0	251.5	6.05
		45	0	254.5	6.32
Soil + 2½ per cent tryptone	*B. subtilis* control	0	20×10^{-5}	.....	6.20
		16	231.0	.....	6.80
		31	1320.0	.....	7.60
		45	1370.0	.....	7.73
	S. griseus control	0	.....	3600.00	7.78
		16	.....	1400.00	7.99
		31	.....	283.3	8.18
		45	.....	304.0	8.11
	B. subtilis + *S. griseus*	0	20×10^{-5}	3600.0	7.78
		16	0	500.0	8.12
		31	0	542.5	7.73
		45	6.2	88.9	7.78

[a] *B. subtilis* was added to the soil 17 days after *S. griseus* and the incubation period refers to the time elapsed from the initial infection with the bacterium.

The pH changes in the soils were rather large, particularly in the presence of the tryptone. In the laboratory, the growth of *B. subtilis* is not adversely affected by pH extremes of 5.9 and 8.2 such as were found in the soil cultures. Therefore, the inhibition of the bacterium cannot be ascribed to changes in pH.

No antibiotic activity could be detected in soil when moist pads of soil from cultures in which *S. griseus* had grown for 45 days were placed on agar plates seeded with a strain of *B. subtilis* that was sensitive to 1 μg. of streptomycin per ml. In addition, water extracts of these cultures were

inactive. Although the cultural studies might indicate that *S. griseus* had produced an antibiotic which had inhibited the growth of *B. subtilis* in the soil, this inference needed further proof for two reasons: first, streptomycin was not extracted from, or its presence demonstrated in, soils seeded with *S. griseus;* and second, additions of the antibiotic to soil in concentrations as high as 500 μg. per gm. failed to inhibit the growth of the bacterium. Furthermore, if streptomycin had been produced in the amended soil (Table 1), it should not have allowed the population of *B. subtilis* to increase as the population of *S. griseus* decreased. Therefore, an alternative hypothesis was tested; namely, that the inhibition of *B. subtilis* was due not to streptomycin but to other antagonistic properties of the actinomycete. For this purpose a number of X-ray mutants of *S. griseus* were

TABLE 2.—*The growth of Bacillus subtilis in a soil in the presence of a non-streptomycin-producing strain of Streptomyces griseus*

Culture	Incubation period[a] (days)	Number of viable cells, millions per gm. of soil		pH
		B. subtilis	RM 3380	
B. subtilis control	0	37.5×10^{-5}	.....	6.20
	9	37.0	.....	6.00
	18	10.7	.....	6.32
	31	54.0	.....	6.69
RM 3380 control	0	.....	705.0	6.25
	9	.....	1280.0	6.37
	18	.....	1490.0	6.74
	31	.....	925.0	7.05
B. subtilis + RM 3380	0	37.5×10^{-5}	705.0	6.25
	9	0	970.0	6.59
	18	0	1635.0	7.05
	31	0	850.0	7.05

[a] *B. subtilis* was added to the soil 17 days after *S. griseus* strain RM 3380 and the incubation period refers to the time elapsed from initial infection with the bacterium.

tested and one of these, RM 3380, had lost its capacity to produce the antibiotic.[2] This characteristic was very stable even after the organism had been cultured in soil many times.

A series of unamended soils was infested with RM 3380 and *B. subtilis* in the manner described for the previous experiments. The data (Table 2) indicate that once again *B. subtilis* was inhibited in the presence of *S. griseus*, but this time by a strain of the organism which did not produce the antibiotic.

PHYSICO-CHEMICAL STUDIES

Though the biological studies indicated that active streptomycin was not necessary for the suppression of *B. subtilis* in the soil cultures, they did not rule out the possibility that the antibiotic might have been produced and then inactivated by the soil. For this reason, it was pertinent

[2] These cultures were obtained through the courtesy of Dr. George Savage of the Upjohn Company, Kalamazoo, Michigan.

to study the effect of the soil on the activity of the antibiotic, and the nature of the effect. The basic character of the streptomycin suggests that it might have a high degree of surface activity towards negatively charged colloidal substances in the soil. Clays or claylike materials comprise a considerable portion of such colloidal complexes; therefore, adsorption studies were carried out on two clay types, one a Wyoming bentonite and the other an illite-type coarse Maquoketa shale colloid,[3] using crystalline streptomycin sulfate assaying 650 units per mg. Both clays are of the 2 : 1 type, that is, the crystal lattice is composed of two plates of silica to one of alumina. However, the bentonite is an expanding clay through which water freely passes, whereas the illite is fixed, the latticework being held together by sheets of K^+ ions. The presence of negatively charged spots on the crystal lattice is due to ionic substitutions within the lattice that cause electrical deficiencies and to unsatisfied valence bonds result-

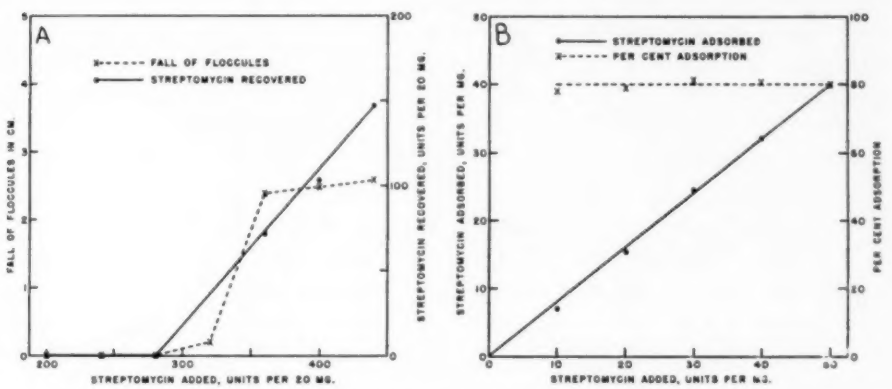

FIG. 1. The adsorption of streptomycin by (A) an illite-type clay, and (B) a bentonite clay.

ing from breakage of the crystal edges; thus, clay minerals are able to enter into base-exchange reactions. Ionic substitutions are much more pronounced in bentonite than in illite; thus the former has a greater number of exchange spots to which cations are attracted and, consequently, a higher base-exchange capacity.

Both clays were diluted in distilled water to concentrations of 4 mg. of clay per ml. Homogenous 5-ml. samples (20 mg.) were pipetted into small vials and various amounts of antibiotic were added to these aliquots. After 5 min., a pronounced visible flocculation of the illite occurred in those vials to which 320 units and more of streptomycin had been added. The distance of fall of the floccules was measured. The suspensions were then centrifuged and the supernatant liquids were assayed for free streptomycin. All assays for streptomycin were conducted by the paper-disc method of Loo et al. (10).

[3] Both clays were obtained from Dr. Roger Bray, Division of Soil Fertility, University of Illinois.

The relationship between flocculation and the appearance of unadsorbed streptomycin in solution is given in figure 1, A. Streptomycin continued to be adsorbed by the clay until all the exchange spots had been occupied by the antibiotic. At this point, the base-exchange capacity of the illite, the colloid flocculated and unadsorbed streptomycin remained in the solution. Three concentrations of the antibiotic, 360, 400, and 440 units, gave readable assays. The quantity of adsorbed antibiotic was determined by subtracting the streptomycin remaining in solution from the total amount added. The values were averaged and the streptomycin adsorption capacity of the illite was computed to be 14.5 units per mg.

When streptomycin is adsorbed on bentonite at concentrations below the total flocculation point, it decreases the light-transmitting power of the suspension. The relation between adsorption of the antibiotic and light transmission was determined by adding various concentrations of streptomycin to a bentonite suspension and diluting to 5.5 ml. These vials were shaken uniformly and then allowed to rest for 15 min., after which the light transmission was measured in a photolometer. The suspensions were then centrifuged and the supernatant liquid was assayed for streptomycin. With increasing adsorption of the antibiotic, there was a linear decrease in light transmission. From the curve in figure 2, B, it is apparent that the amount of streptomycin adsorbed is proportional to its concentration. An adsorption constant K equal to 0.8 was obtained, indicating that at concentrations below the flocculation point about 80 per cent of the antibiotic added was adsorbed. To determine whether this adsorption constant was due to an equilibrium phenomenon or simply a time-rate reaction, a suspension of bentonite was shaken with streptomycin for 24 hr.; at various time intervals, aliquots were removed and assayed for free streptomycin. No change in adsorption could be detected during the time of contact. Apparently, the adsorption phenomenon involves an equilibrium between the antibiotic and cations already adsorbed onto the exchange spots of the clay.

To ascertain whether true adsorption was responsible for the disappearance of streptomycin from the solvent phase, X-ray diffraction studies were made.[4] A spread of the crystal lattice indicates adsorption. At 80 units of streptomycin per mg. of bentonite, this clay was completely flocculated. The flocculated material was filtered and dried for X-ray diffraction studies using an iron K alpha emission line. The spread obtained, as measured by the 001 spacing, of 15.17 Ångstrom units is considerably larger than 13.0 Å obtained for the untreated clay. This significant difference of 2.17 Å demonstrates that streptomycin was adsorbed.

Although it was established that streptomycin could be adsorbed, this did not reveal whether such adsorbed antibiotic could enter into normal base-exchange reactions. If such a dynamic situation could exist in the soil, any streptomycin produced there could eventually act upon the sus-

[4] The X-ray studies were carried out by Mr. Max Mortland, Division of Soil Physics, University of Illinois.

ceptible flora after being replaced from the colloidal complex by other cations. To determine the tenacity with which the antibiotic is held on the clay surface, a solution was added to a bentonite suspension to the point of base saturation where flocculation just occurred and no free streptomycin remained in the solution. The suspensions were then shaken for 2 hr. with a number of base-exchange agents, including the hydrogen ion, methylene blue, and janus green. The supernatant liquid was then assayed for displaced streptomycin. Another suspension was electrodialyzed for 24 hr. and samples were removed at various intervals for assay. Only methylene blue and janus green were able to replace adsorbed streptomycin to any extent. The ring sizes of the inhibition zones developed on the *B. subtilis* plates indicated that the antibiotic is very strongly bound; only about 5 per cent of the adsorbed antibiotic was recovered. No streptomycin could be recovered from the clay by electrodialysis. Because streptomycin is difficult to replace, one must conclude that this antibiotic is firmly bound by clay.

TABLE 3.—*The removal of streptomycin from solution by six natural substrates*

Substrate	Streptomycin adsorbed, units per gm.	Adsorption capacity[a]
Acid-washed quartz	416.0	4.7
Sandy soil	8866.0	100.0
Brown loam	9775.0	110.0
Black clay loam	9960.0	112.0
Michigan muck	9915.0	111.0
Maquoketa shale colloid (illite)	14500.0	163.5

[a] Based on an arbitrary standard of 100 for the sandy soil.

The adsorption of streptomycin occurring with isolated clays also takes place in field soils, indicating that even if it were produced by *S. griseus*, the antibiotic would be removed from the soil solution. The adsorption capacities of several soil types and an acid-washed quartz were determined. The soils were first brought to 100 per cent moisture-holding capacity and then shaken for 2 hr. with 10,000 units of streptomycin in 5-ml. solutions. Assays for the active antibiotic were carried out as described for the studies with clays. The adsorption capacities were compared with that of the coarse shale colloid (Table 3). Coarse sand had very little adsorptive capacity. The three mineral soils which contain clay had high capacities. It is interesting that the muck which contains practically no clay was highly adsorptive, indicating that soil organic matter also plays a role in removing streptomycin.

Base-exchange procedures failed to remove streptomycin from the soil complexes except for one case: 1 per cent methylene blue removed 7.5 per cent of the antibiotic adsorbed by the sandy soil. Standard procedures for maltol determination as a chemical assay for streptomycin were attempted on soils treated with 2,000 units of streptomycin per gm., but only

very faint color reactions were obtained (17, pp. 74–91). Even at such a high concentration only a very small amount of the antibiotic could be detected. It is extremely doubtful that *S. griseus* could produce detectable amounts of streptomycin in the soil.

The possibility still remained that streptomycin might be biologically active in an adsorbed state and could inhibit the growth of bacteria in the soil. Sterilized black clay-loam soils were treated with sterile streptomycin to give concentrations of 0, 50, 250, and 500 units per gm. The soils were brought to optimum moisture content, and then were infested with *B. subtilis*. After a 72-hr. incubation period at 28° C. the soils were plated. No differences were observed in the ability of *B. subtilis* to flourish in the control and treated soils, proving that adsorbed streptomycin in the soil is inactivated.

DISCUSSION

The biological studies demonstrated that some form of antagonism developed between *S. griseus* and *B. subtilis* to the detriment of the bacterium. This antagonism could not be the result of the secretion of streptomycin since it is inactivated in the soil by adsorption which, in this case, is highly irreversible. Furthermore, a similar antagonism was demonstrated by a nonstreptomycin-producing mutant. The inhibitive effect might have been due to competition for food or for space. This is borne out by the fact that in an equal period in unamended soil, the actinomycete produced a population about ten times as great as that of the bacterium; apparently, *S. griseus* found the soil a more favorable growth medium than did *B. subtilis*. In tryptone-treated soils the bacterium multiplied about as well as the antagonist when each was grown alone. However, when *B. subtilis* was added to the amended soil after *S. griseus* already was well established, the bacterium failed to reproduce until the population of the actinomycete had passed its growth peak and dropped to 89 million cells per gm. of soil. This closely parallels the situation in the untreated soils, where the susceptible organism appeared when the population of the antagonist was only 84 million cells and then disappeared as the numbers of the actinomycete rose. If an active antibiotic had been elaborated in the soils, we should except a complete "liquidation" of *B. subtilis* and no initial or subsequent development of a bacterial population, though it is possible that streptomycin-resistant mutants may have developed. If this actually occurred, then it is difficult to understand the sudden disappearance and appearance of *B. subtilis;* instead, a steady rise in population would occur as the mutants established themselves.

Many of the antibiotic substances produced by antagonists such as streptomycin, streptothricin, tyrothricin, subtilin, etc., are basic in nature. If such agents are actually produced in the soil, then the effect of the soil upon their activity must be evaluated. The work of Gieseking (4) has shown that large-moleculed basic substances are adsorbed by clays with a

high degree of irreversibility. This irreversibility may be due, in part, to the high energy of adsorption of such molecules and to steric hindrance as the molecules expand the crystal lattice and pack the interstices. The base-exchange studies with streptomycin confirm Gieseking's results that large complex cations are exchangeable by other cations of approximately the same size but not by hydrogen, which is very effective in replacing small cations (4). Actually, only a small percentage of the adsorbed streptomycin could be replaced by the dye molecules, methylene blue or janus green, possibly because the molecules of the antibiotic are so oriented and packed into the interstices as to prevent the entrance of the dye molecules. In all probability, only the molecules at the exposed surfaces of the clay can be replaced.

The streptomycin molecule is an extended structure whose basicity is primarily derived from the guanido components in the streptidine group and also from the N-methyl glucosamine. In solution, the ionization of the molecule creates a number of charged spots, each of which can act as an adsorption center for collodial particles. Thus, a large heavy matrix is formed and the mass flocculates out. From such a point of view, streptomycin acts as a trivalent ion with a low zeta potential.

On the basis of both the biological and adsorption studies, one can conclude that streptomycin is not active in influencing the microbiological population of the soil and that the inhibition of *B. subtilis* by *S. griseus* was not due to the presence of the antibiotic. The results do not preclude the production of streptomycin under natural soil conditions, but even then, the antibiotic is so rapidly adsorbed and thereby inactivated by the soil complex that probably it has no significant effect on normal biological equilibria. From our knowledge of adsorption phenomena in soils, we can assume that many of the basic antibiotics will undergo the same fate as streptomycin.

SUMMARY

Both a streptomycin-producing strain and a nonproducing mutant of *S. griseus* depressed the development of *B. subtilis* in unamended and tryptone-amended soil cultures.

Colloidal complexes such as clays and soil organic matter adsorb and effectively inactivate streptomycin.

It is highly probable that basic antibiotics such as streptomycin do not play an active biological role in the soil.

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PHYSIOLOGICALLY ACTIVE EMANATIONS FROM VIRUS–INFECTED PLANTS

A. Frank Ross and C. E. Williamson

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The symptoms developed by plants of *Physalis floridana* Rydb. infected with potato virus Y (*Marmor upsilon* H.) (16) are to some extent similar to those of certain plants exposed to ethylene. About 10 days following inoculation, the inoculated leaves show epinasty (Fig. 1) and a few days later they abscise. Leaves infected by systemic spread of the virus also become epinastic and eventually abscise. Because of this similarity in symptomatology and the fact that Williamson (18) found that plants infected by certain fungi produce abnormally large amounts of ethylene, tests were made for physiologically active emanations from Y-infected plants of *P. floridana*. Data showing that abnormal amounts of physiologically active emanations, presumably ethylene, are produced by such plants are presented in this report, together with similar data on other viruses in various suscepts.

The leaves of several plant species apparently produce ethylene as a normal metabolic product (3, 4, 18), but no report has been found in the literature of a marked stimulation of ethylene production following virus infection. Grieve (7, 8) reported epinasty as a primary symptom of rose-wilt virus (*Marmor flaccumfaciens* H.) and noted a strong tendency toward epinasty in tomato plants infected with tomato spotted wilt virus (*Lethum australiense* H.). While ethylene was not implicated, Grieve (7) did comment on the similarity in appearance of infected rose plants and rose plants exposed to ethylene. Since ethylene and auxin have been reported to be antagonistic (1), Grieve's finding that infection by either virus was accompanied by a partial destruction of growth hormones (7, 9) does not exclude the possibility that ethylene was the primary cause of the epinastic condition.

EXPERIMENTAL WORK

Detection of Ethylene

Etiolated pea (*Pisum sativum* var. Alaska) seedlings, prepared and grown under standard conditions (15), were used for the detection of ethylene. The procedure described by Williamson (18) was followed in all experiments. Unless otherwise stated, 15-gm. samples of leaf tissue were used and all tests were made at 70° F. Relative amounts of ethylene produced by different types of tissues were estimated from the extent of the triple response, defined as a change of negative geotropism to diageotropism, increased growth in thickness, and reduced rate of growth in length (12).

Potato Virus Y in Physalis floridana

The strain of potato virus Y used and the methods by which its freedom from other viruses was established have been described previously (17).

Inoculated leaves with necrotic lesions. All of the mature or nearly mature leaves of several large plants of *Physalis floridana* were dusted with carborundum and rubbed with a gauze pad saturated with a 1:5 dilution (0.1 M glycylglycine buffer pH7) of freshly expressed juice of diseased plants (*Nicotiana tabacum* L. or *N. glutinosa* L.). The leaves of a like number of comparable plants were treated similarly except that buffer solution was substituted for the diluted virus-containing plant juice. Ten to 12 days later, or as soon as lesions had developed on those leaves inoculated with virus Y, leaves from each group were collected and enclosed with the pea seedlings under battery jars. Care was taken in selecting the leaves so that those comprising a given diseased sample and its corresponding healthy control were as nearly alike as possible in all other respects. Approximately 500 lesions developed on each of the Y-inoculated leaves.

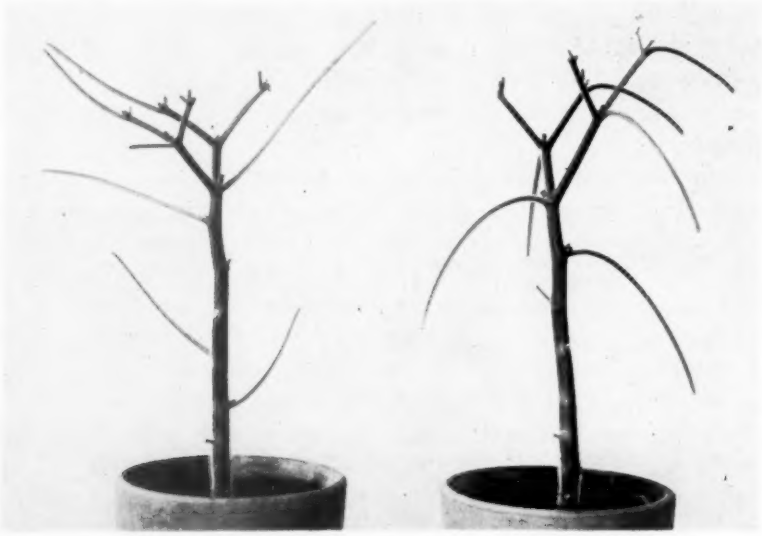

FIG. 1. Epinasty in plants of *Physalis floridana* infected with potato virus Y. Healthy plant on left; plant on right was inoculated 11 days previously. All leaves were removed so that the epinastic petioles can easily be seen.

Intact leaves from the healthy plants produced no more than a trace of ethylene, whereas comparable ones from diseased plants produced appreciable amounts (Table 1, Fig. 2). The intensity of the response of the seedlings to emanations from the virus-infected leaves was comparable to that of emanations from leaves infected with certain fungi recorded by Williamson (18). Shredding of healthy leaves of *P. floridana* did not cause any observable increase in evolution of ethylene, a phenomenon reported by Williamson for certain other types of healthy plant leaves. There appeared to be no correlation between the amount of ethylene produced and the age of leaf used. The response to emanations from leaves having marked epinasty was, in general, greater than from leaves less epinastic.

Y-infected leaves with no necrosis. At 80° F. or above, leaves of *P.*

floridana inoculated with virus Y become infected without development of necrotic local lesions (16). Although such leaves are symptomless, their virus content is much higher than that of comparable leaves grown at temperatures that permit lesion development (17). It was of interest to determine whether ethylene production is associated with the development of necrosis or with a high rate of virus multiplication.

Several plants were inoculated, by the methods described, with diluted juice containing virus Y. A like number were rubbed with buffer solution. Half of each lot was placed in a greenhouse room maintained at 80° F. or above, and the other half in a room maintained near 70° F. Numerous lesions developed on the Y-inoculated leaves of the latter group and none on any of the other leaves. Leaves from each group were tested for the

TABLE 1.—*Response of etiolated pea seedlings to emanations from healthy leaves of Physalis floridana and from similar leaves 10 days following inoculation with potato virus Y*

Test	Type of tissue	Response rating[a] to emanations from	
		Healthy leaves	Diseased leaves
1	Intact	1	5
	Do	1	4
	Do	1	4
	Do	1	4
2[c]	Intact	0	5
	Shredded[b]	0	
	Intact	1	4
	Intact	0	4
	Shredded[b]	0	
	Intact	0	4

[a] Response of pea seedlings rated from zero through five. A rating of 5 indicates no elongation, only a clublike swelling of the growing point.
[b] Leaves finely shredded with a sharp knife just prior to enclosure with seedlings.
[c] A no-tissue control gave a zero response.

emanation of ethylene by the usual procedure at 70° F. The ratings of the response to emanations from necrotic infected leaves varied from 3 to 4 and those for nonnecrotic infected leaves varied from 0 to 2. In simultaneous tests at 80° F., the ratings were 2 to 3 and 1, respectively. At both temperatures, the ratings for healthy leaves were never higher than one. Thus ethylene production was highest in those leaves with necrosis and not in the leaves highest in virus content.

Other Viruses

Inoculated leaves with necrotic lesions. A few tests were made with other viruses that produce local lesions on inoculated leaves. These viruses were tobacco-mosaic virus (*Marmor tabaci* H.), alfalfa-mosaic virus (*M. medicaginis* H.), tobacco ring-spot virus (*Annulus tabaci* H.), and potato

virus X (*A. dubius* H.). All viruses were of proved identity and purity except that referred to as alfalfa-mosaic virus. This virus was isolated from alfalfa and while its symptomatology differs from that of the type strain, its host range and other properties are similar.

In all cases, leaves were inoculated with inocula calculated to produce very large numbers of lesions but not concentrated enough to cause general collapse or death of the leaves. The leaves were tested for ethylene emanation soon after the appearance of necrosis but before the lesions had reached maximum size.

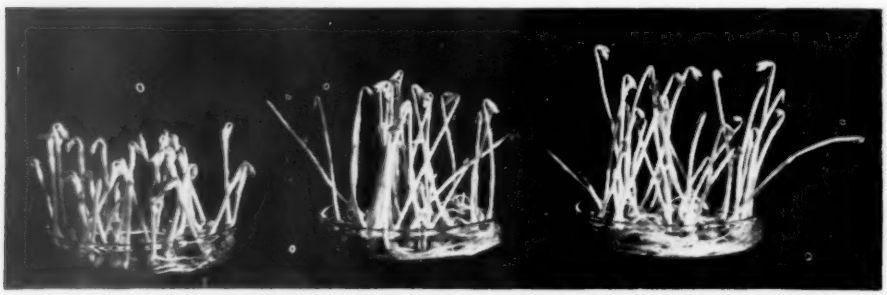

FIG. 2. Etiolated Alaska pea seedlings after 72-hr. exposure at 70° F. to emanations from 15 gm. of leaves of *Physalis floridana* (left to right): inoculated with potato virus Y, intact healthy leaves, and shredded healthy leaves.

In all cases but two, the diseased leaves produced greater amounts of ethylene than did corresponding healthy leaves (Table 2). The two exceptions were tobacco-mosaic virus in leaves of Scotia bean and potato virus X in Turkish tobacco leaves. That the failures were not due to the peculiarity of those plants is shown by the facts that healthy controls produced detectable amounts of ethylene and that leaves of these same plants infected with other viruses produced large amounts of ethylene. In both exceptions lesions were very small, i.e., necrosis was not nearly so extensive as in the other cases. When old plants of *N. glutinosa* were used as suscept for tobacco-mosaic virus, the lesions, and the amount of ethylene produced, were small. Hence, in general, the amount of ethylene produced was roughly proportional to the extent of necrosis.

Systemically infected leaves. A few tests were made with noninoculated leaves of systemically infected tobacco plants. The viruses selected produced either green mosaics, yellow mosaics, or necrosis in the noninoculated leaves. In general, the diseased leaves tended to produce slightly greater amounts of ethylene than did corresponding healthy ones. However, the behavior of both types of leaves was so variable that no definite conclusions could be made. Whether this variability was attributable to the use of young, rapidly growing leaves, or to seasonal or other factors, has not yet been determined.

Phytotoxic Chemicals

Leaves of *Physalis floridana* and of *Nicotiana tabacum* with necrotic lesions induced by phytotoxic chemicals, e.g. copper sulfate, also produced

TABLE 2.—*Response of etiolated pea seedlings to emanations from leaves inoculated with different viruses*

Virus	Suscept	Type of material[a]	Response rating[b] to emanations from	
			Healthy leaves	Diseased leaves
Tobacco mosaic	*Nicotiana glutinosa*	MP, UL	1	4
		MP, LL	0	3
		LP, RS	0	1
		LP, RS	0[c]	1
	Datura stramonium	MP, RS	2	5
		MP, RS	3[c]	5
	Phaseolus vulgaris var. Scotia	TL	0	2
		TL	2	2
		TL	1	1
Alfalfa mosaic	*Phaseolus vulgaris* var. Scotia	TL	2	4
		TL	1	4
Tobacco ring spot	*Nicotiana tabacum* var. Turkish	MP, RS	1	4
		MP, RS	0[c]	5
Virus Y	*Solanum tuberosum* (seedling 41956)	LP, RS	0	4
		LP, RS	2[c]	4
Virus X	*Gomphrena globosa*	LP, TL	1	5
		LP, TL	1[c]	4
	Nicotiana tabacum var. Turkish	YP, RS	1	1
		YP, RS	2[c]	1

[a] Symbols in this column: YP—young plants with less than 6 leaves; MP—medium-sized plants not yet flowering; LP—large plants in flowering stage; UL—upper leaves; ML—middle leaves; LL—lower leaves; TL—first true leaves; RS—leaves selected at random.

[b] See footnote a, table 1.

[c] Leaves shredded with sharp knife just prior to test.

ethylene. The amounts detected were but slightly smaller than those from comparable leaves with lesions induced by viruses. This is taken as evidence that ethylene is a product of dying or necrotic cells, rather than the cause of necrosis.

DISCUSSION

Ethylene, propylene, acetylene, and carbon monoxide cause the triple response in pea seedlings (12) and epinasty of leaves of certain plants (2). Hence, the physiologically active emanations detected in these experiments could be any one or a mixture of these materials. Ethylene is the only one that is effective in very low concentration. In all cases where an active emanation from plant tissues has been identified chemically, it has proved to be ethylene (6, 10, 11, 13, 14). Hence, the emanation from virus-infected leaves is presumed to be ethylene.

Production of appreciable quantities of ethylene by infected leaves does not necessarily mean that such leaves will become epinastic. Epinasty has been noted in all of the suscept-virus combinations in table 2 except those involving *Nicotiana tabacum* and *Phaseolus vulgaris*. According to Crocker, Zimmerman, and Hitchcock (2), exposure to ethylene does not in-

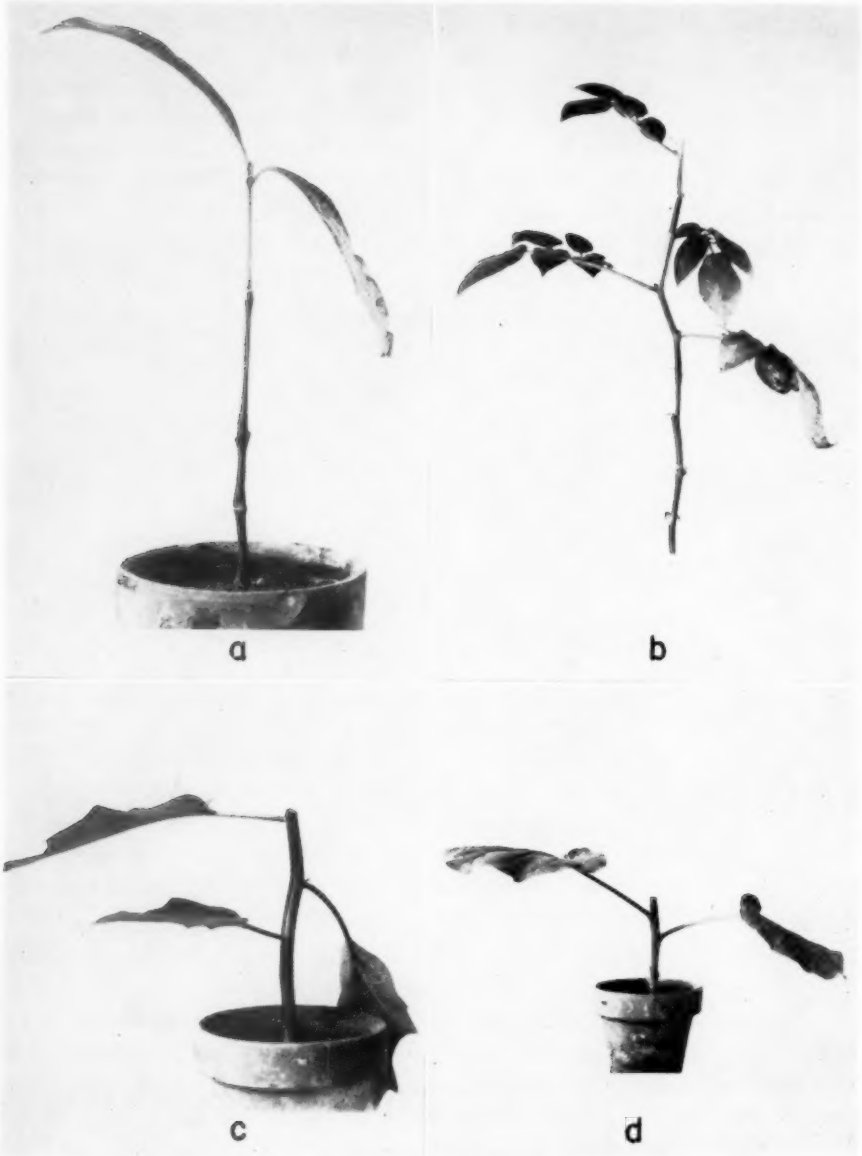

FIG. 3. Epinasty of virus-infected leaves. In each case only those leaves on the right were inoculated. (a) Potato virus X in *Gomphrena globosa;* (b) potato virus Y in U. S. Dept. Agr. potato seedling 41956; (c) tobacco-mosaic virus in *Datura stramonium;* (d) tobacco-mosaic virus in *Nicotiana glutinosa.* Plants were decapitated and all leaves but those shown were removed just prior to photography.

duce epinasty in these two species, whereas exposure of *N. glutinosa* induces slight epinasty and exposure of *Gomphrena globosa* and *Solanum tuberosum* induces marked epinasty. Doubt (5) reported that leaves of *Datura stramonium* became epinastic following exposure to ethylene. In

the present tests, epinasty was slight in *N. glutinosa* and marked in *G. globosa*, *D. stramonium*, and *S. tuberosum* (Fig. 3).

Since leaves with necrosis induced by nonpathogenic agents produced abnormal amounts of ethylene, necrosis would appear to be the cause rather than the result of ethylene production. On the other hand, certain other commonly observed virus symptoms may be the direct result of abnormal production of ethylene. Epinasty and abscission of leaves probably are related directly to ethylene production. In addition, there are a number of other symptoms common to some virus diseases and to ethylene toxicity. These include chlorosis, hyperplasia, retardation of growth, and abscission of petals or flower buds. This is not to suggest that all such symptoms in virus-infected plants are due to ethylene, but it appears possible that in certain cases such symptoms may be the direct result of the production of abnormal amounts of ethylene by virus-infected plants.

SUMMARY

Leaves of several plant species inoculated with certain viruses produced a physiologically active emanation in amounts much greater than that produced by comparable healthy leaves. The emanation, presumably ethylene, was detected and measured biologically by the triple response of etiolated pea seedlings. Ethylene production appeared to be the result of necrosis and not the cause. It was greatest in leaves inoculated with viruses that cause numerous large necrotic lesions. In plants of *Physalis floridana* inoculated with potato virus Y, ethylene production was much greater at 70° F., a temperature favorable to the development of necrosis, than at 80° F., a temperature favorable to virus multiplication without the development of necrosis. Epinasty was noted in those suscept-virus combinations in which large amounts of ethylene were detected, provided epinasty is a normal response of that plant species to exposure to ethylene.

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FUNGI ASSOCIATED WITH THE ROOTS OF LITTLELEAF-DISEASED AND HEALTHY SHORTLEAF PINE

W. A. CAMPBELL[1]

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Littleleaf is a common disease of shortleaf pine (*Pinus echinata* Mill.) and, to a lesser extent, of loblolly pine (*Pinus taeda* L.) from western Mississippi throughout the Southeast to Virginia. It occurs mainly on piedmont soils and on closely associated upper coastal plain and mountain soils. The symptoms, characterized by a progressive decline of the top, have been described in detail by Hepting, Buchanan, and Jackson (7). The disease is confined for the most part to stands older than 20 years and has caused millions of dollars' loss in the Southeast in the past ten years.

Siggers and Doak (9), who first investigated littleleaf disease in Alabama, noted that the roots of diseased trees were defective, many of the finer roots being dead or missing; larger living roots had shallow cortical lesions and occasional pitchy cankers. Although they made 2000 isolations from diseased roots, mostly of $\frac{1}{4}$-in. diameter or larger, no fungus recognized as a pathogen was consistently associated with any of the root abnormalities. However, a slow-growing fungus with black mycelium was commonly present in the cortical lesions.

Jackson (8) continued the investigation of root defects, especially the cortical lesions, which he termed "brown patch" and which occurred on healthy as well as diseased trees. He named the slow-growing black fungus *Torula marginata* L. W. R. Jacks, and concluded that it was consistently associated with "brown patch." His inoculations with *T. marginata* caused only limited lesions on large roots of healthy trees, and inoculum applied in quantity to the soil near roots of pole-size healthy trees failed to cause littleleaf.

Jackson also discovered the association of sclerotia of *Poria cocos* Wolf with the roots of littleleaf trees in several areas. However, the fungus was not consistently associated with littleleaf trees in most areas and sclerotia were also found on healthy trees.

By the end of 1945, extensive investigations on the pathology of larger roots of shortleaf pine had not revealed any recognized pathogens that could be considered likely causes of littleleaf. The possibility still remained that some undiscovered fungus was the primary agent in necrosis of fine roots. To determine whether or not pathogens were associated with these roots, a study of the fungi associated with mycorrhizal and fine roots was started in September 1946. Two methods of isolation were used: 1) a

[1] The writer is especially indebted to Ralph Emerson for identifying a number of the fungi found in this study. Thanks are also extended to W. L. White, Kenneth B. Raper, Ross W. Davidson, David R. Sumstine, Lawrence W. Ames, and Julian H. Miller for their assistance.

general method designed to recover slow-growing as well as fast-growing organisms, and 2) special isolation methods for the recovery of water molds.

GENERAL ISOLATION METHOD

From September 1946 to March 1948, isolations were made from the fine roots of 74 littleleaf trees, 24 healthy trees on littleleaf sites, and 25 healthy trees on littleleaf-free sites. The majority of the samples from littleleaf sites were collected near Dadeville, Alabama, Walhalla and Union, South Carolina, and Elberton and Athens, Georgia. Most of the root samples from healthy trees on littleleaf-free sites were taken near Asheville and Durham, North Carolina, in areas generally free of littleleaf.

For isolation purposes, four categories or types of root sample material were recognized: 1) dead roots, preferably those that had been dead but a short time, 2) root pieces in which dead and living tissues were sharply defined so that isolations could be made from adjacent living and dead material, 3) living roots, and 4) mycorrhizal clusters. Isolations were made chiefly from roots $\frac{1}{8}$ in. in diameter or smaller.

Soil type and condition were important factors in obtaining satisfactory root samples for isolations. In general, small roots and mycorrhizae could be collected easily from sandy soils, less readily from loams, and with great difficulty from clays. The relative abundance of fine roots and mycorrhizae varied according to the condition of the tree; they were characteristically few, or lacking almost entirely, from trees with advanced littleleaf.

Approximately 26 duplicate samples of root material were selected for culturing from each tree. The samples were surface-treated in 30 per cent hydrogen peroxide, one piece being immersed momentarily and the other treated for from 4 to 10 sec., depending upon the size. The excess peroxide was drained off and the samples were placed between a block of water agar and the glass in a Petri plate, and incubated at room temperature (approximately 76° F.).

Mycelial growth from root pieces was observed under the binocular dissecting microscope. When such growth extended through the agar or had advanced a short distance from the inoculum, a small bit of agar and mycelium was transferred to potato-dextrose or malt agar in a test tube. By this technique several fungi could be recovered, in pure culture, from a single piece of root. If fast-growing species such as *Trichoderma* were present, slower-growing fungi could be recovered by removing single hyphal tips under the dissecting microscope.

The fungi isolated from the roots of healthy and littleleaf-diseased shortleaf pine fell into 11 groups (Table 1). Nine of these groups were represented by single species, or species of closely related genera. Most of the groups include fungi known to be nonpathogenic. Species of *Trichoderma* (chiefly *Trichoderma viride* (Fr.) Harz) and *Gliocladium* were associated with the roots of all trees, regardless of condition. No attempt was made to identify most of these as to species because they were common soil fungi

and not considered pathogenic. Although a considerable number of different species of both *Penicillium* and *Aspergillus* were isolated, only *Aspergillus flavus* Link, *A. niger* van Tiegh., *Penicillium spinulosum* Thom, *A. janthinellum* Biourge, and *P. javanioum* Kingma were identified as to species. The difficulty involved in getting specific identification of the others was considered to be out of proportion to their possible relation to littleleaf disease.

Monotospora sp., *Mortierella* sp., and the unidentified dark fungus were considered nonpathogenic. It is interesting to note that these fungi and the unidentified Phycomycete were most frequently isolated from healthy trees on littleleaf-free sites.

TABLE 1.—*Fungi isolated from the small roots[a] of healthy and littleleaf-diseased shortleaf pine*

Fungi	Diseased trees on littleleaf sites		Healthy trees on littleleaf sites		Healthy trees on littleleaf-free sites	
	Per 100 samples	Trees isolated from	Per 100 samples	Trees isolated from	Per 100 samples	Trees isolated from
	Number	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*
Trichoderma and *Gliocladium* spp.	39	100	47	100	53	100
Penicillium and *Aspergillus* spp.	35	97	35	92	31	100
Monotospora sp.	19	84	20	96	34	100
Torula marginata	28	96	22	96	18	72
Unidentified dark fungus	12	73	16	79	37	100
Unidentified Phycomycete	8	44	11	100	13	84
Mortierella sp.	2	35	2	25	11	76
Unidentified No. 6	3	34	1	25	1	8
Unidentified No. 10	4	26	3	13	0	0
Miscellaneous nonpathogens	4		5		6	
Unclassified unidentified fungi	18		16		11	
All fungi	174		178		215	
Total trees		74		24		25

[a] Mainly those of ⅛-in. diameter and smaller, including mycorrhizal clusters.

The group containing the miscellaneous nonpathogens includes such identified species as *Pachybasium pyramidale* (Bon.) Cav., *Mortierella turficola* Ling-Young, *Absidia spinosa* Lindner, *Pullularia pullulans* (D By.) Berkhout, *Pestalotia funerea* Desm., *Chaetomium fusiforme* Chivers, and *Rhizopus nigricans* Ehr. In this group were also placed Actinomycetes and unidentified representatives of the following genera: *Mucor, Absidia, Zygorrhynchus, Cunninghamella, Melanopus, Collybia, Coniothyrium, Alternaria, Graphium, Acremonium,* and *Helminthosporium.*

The unclassified unidentified fungi made up 9 per cent of the total isolations and included a variety of types which occurred infrequently and

were, because of their rarity, not suspected of causing littleleaf. Most of them were sterile and produced only a mass of mycelium in culture, and were considered unidentifiable. Judging by clamp connections on the hyphae, some of these were Basidiomycetes, but as they did not fruit in culture they could not be identified. One fungus in this group produced a slow-growing, sterile, black mycelium and was considered to be *Mycelium radicis nigrostrigosum* Hatch.

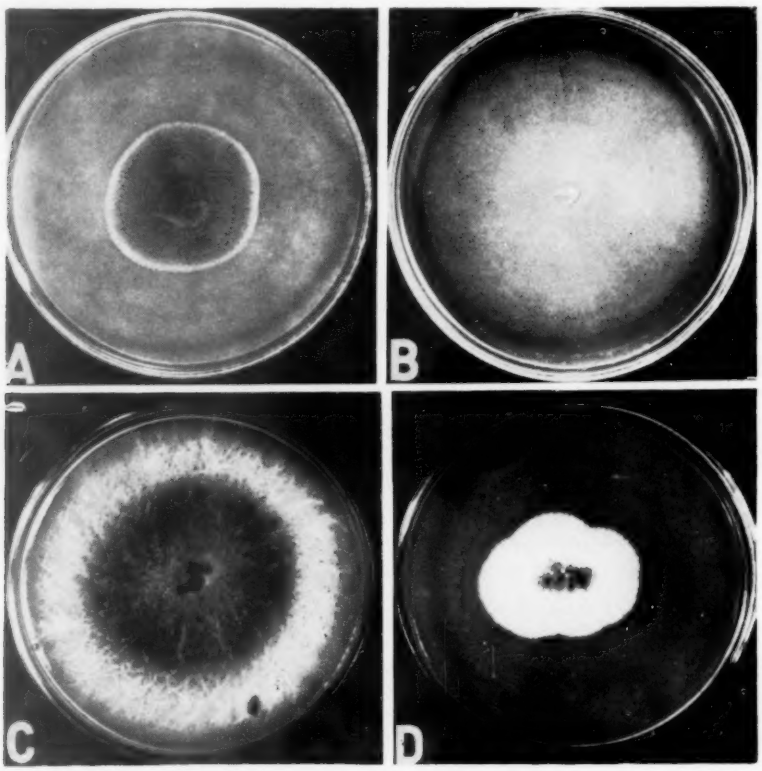

FIG. 1. A. Unidentified dark fungus; mat age 7 days. B. Unidentified Phycomycete; mat age 7 days. C. Unidentified No. 6; mat age 10 days. D. Unidentified No. 10; mat age 10 days. All cultures were grown on malt agar (Difco) in diffused light at room temperature (approximately 76° F.).

Torula marginata, the best suspect, based on past isolations and association with brown patch, is eliminated as a likely primary pathogen because, although more abundant on littleleaf sites, it occurred in only slightly smaller numbers from root samples from disease-free sites. Hepting (6) considered brown patch as premature "rough bark" formation resulting from cork cambium deep in the phloem; he also judged that it occurred particularly in the roots of littleleaf trees because their roots are starved for carbohydrates. *T. marginata* consistently inhabits the excised phloem tissues, apparently living on their remaining cell contents.

Poria cocos was isolated once from a large root on a diseased tree.

Although sclerotia were found occasionally on healthy and diseased trees, the fungus was never isolated from small roots. A few isolations of *Sclerotium bataticola* Taub. were made during the summer months but the fungus was encountered too rarely to be considered associated with littleleaf. *Fusarium* spp. were never isolated from shortleaf roots, an interesting situation in view of the widespread distribution of such fungi on agricultural crops in the region. Only the fungus designated as Unidentified No. 10 was limited to littleleaf sites. However, this fungus has been unable to harm seedlings or large trees in inoculation tests.

Four of the fungi listed in table 1, namely, the unidentified dark fungus, the unidentified Phycomycete, and the unidentified numbers 6 and 10, occurred in sufficient numbers to permit the conclusion that they are common associates of the roots of shortleaf pine. Brief cultural descriptions follow.

Unidentified dark fungus (Fig. 1, A). Growth slow, forming in 10 days a mat 2.5–4 cm. in diameter;[2] mat raised, wooly, homogeneous, dark

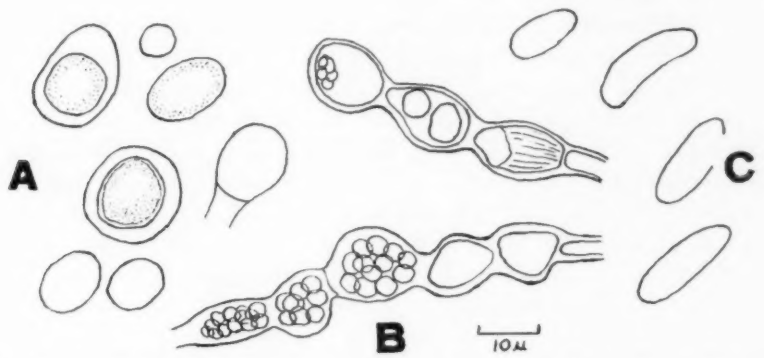

FIG. 2. A. Unidentified Phycomycete; spore forms abundant after cultures are a few days old. B. Unidentified No. 6; inflated chainlike hyphae. C. Unidentified No. 10; spores.

gray or black, with a narrow, colorless, appressed margin; aerial hyphae smooth, dark colored, with few septations, 2–5 μ in diameter; submerged hyphae 2–6 μ in diameter, with swollen cells filled with oily droplets, these released abundantly in crushed mounts, not grouped in torulalike chains, usually light colored but brownish in older portions of mat; older hyphae often covered with fine brownish crystals.

Unidentified Phycomycete (Fig. 1, B, and 2, A). Growth rapid, forming in 7 days a mat 7–9 cm. in diameter; mat thin, practically colorless, without aerial hyphae and with faint to distinct radiating lines; margin colorless, even; hyphae 2–4 μ in diameter, much branched; globose to ellipsoid or irregular-shaped chlamydosporelike bodies formed early, these becoming more abundant with age, 6–20 μ in diameter. After 14 days no

[2] Cultural descriptions based on mats grown on Difco malt agar, at room temperature (approximately 76° F.), in diffused light.

change in the appearance of the mat, chlamydosporelike bodies very abundant with occasionally one developing into a well-formed oospore, no antheridia noted; most of the oosporelike bodies imperfectly formed.

Unidentified No. 6 (Fig. 1, C, and 2, B). Growth moderately rapid, forming in 7 days a mat 4–5 cm. in diameter; mat variable, when first isolated rather thin, appressed, cottony, usually with fimbriate strands, darker at center; margin wide, cottony, fimbriate; hyphae at growing points much branched, 2–7 μ in diameter, septa at frequent intervals with tendency for swollen cells to develop; older hyphae from center of mat a mass of short, swollen cells filled with granular or oily contents, numerous oily droplets freed from macerated cells in crushed mounts; no spores. In 14 days, mat 6–9 cm. in diameter with a dark, slate-colored central zone (sometimes brownish) and a wide white marginal zone; dark central zone a mass of torulalike cells.

Unidentified No. 10 (Fig. 1, D and 2, C). Growth very slow, forming in 7 days a mat 1.5 cm. in diameter; mat when first isolated raised, cottony, white for most part but somewhat tan or pink at center, underside tannish around inoculum; hyphae smooth to irregular, often much twisted and contorted, filled with oily droplets, colorless, 2–8 μ diameter; large crystals common. In 14 days, mat 2.5–3.5 cm. in diameter; raised, cottony or wooly, mostly with irregular margins and a strong tendency to sector; color variable from white to tan; underside of mat tannish or pinkish; spores common in cultures 20 days old, single-celled, rounded at both ends, straight or slightly curved, 5–8 $\times$ 15–30 μ, borne singly or on conidiophores grouped in masses.

WATER MOLD ISOLATION METHODS

The general isolation method failed to yield species of *Phytophthora* or common species of *Pythium*. Crandall (5) has demonstrated that special handling is sometimes necessary to isolate *Phytophthora cinnamomi* Rands from root material. Therefore an effort was made during March and April, 1948, to obtain this fungus (1), which was known to occur on chestnut in the same area as littleleaf (4), and other water molds by incubating root pieces in distilled water for 3 to 5 days before plating on cornmeal agar. Root samples from 16 littleleaf trees were so treated. *P. cinnamomi* was isolated from the roots of one tree, and an unidentified *Phytophthora* from another. A species of *Endogone* was also commonly recovered. Several Pythiumlike fungi were also isolated but these were not identified. In general, the same fungi were obtained after root pieces were soaked in distilled water as by the general isolation method. However, slow-growing forms were less frequently isolated because the faster-growing ones were favored by the isolation method.

From July to November 1948 the search for *P. cinnamomi* was continued, chiefly by the use of Tucker's apple technique (10). During this period root samples were taken from 28 littleleaf-diseased trees. *P. cinnamomi* was isolated from approximately 2 per cent of the root samples

from 11 of the trees. The fungus was not obtained from the other 17 trees.

Further efforts to isolate *P. cinnamomi* from the roots of littleleaf trees demonstrated that the fungus could be recovered only from recently killed small roots and mycorrhizal clusters. Apparently the fungus is active in a small root for only a short time, or on a narrow advancing margin in a root, before it is superseded by saprophytic fungi. The chances of finding small roots in the proper stage of infection under littleleaf trees for the isolation of *P. cinnamomi* are rather remote, in view of the dearth of fine roots under such trees. The tendency for *P. cinnamomi* to attack only fine roots has been demonstrated in a preliminary inoculation experiment with shortleaf pine seedlings. The fungus could be isolated without difficulty from the water-soaked ends of new roots. Only saprophytes could be isolated from such material after it had become brown or softened. With the development of a technique for making isolations directly from the soil (2), root isolations were abandoned in favor of this more rapid method of determining the relative abundance of *P. cinnamomi* in the root zones of healthy and diseased trees (3). To date *P. cinnamomi* is the only known parasitic fungus to be isolated from the roots of littleleaf-diseased shortleaf pine.

SUMMARY

Root samples were taken from 74 littleleaf trees, 24 healthy trees on littleleaf sites, and 25 healthy trees on littleleaf-free sites. By the use of a standard isolation technique fungi were isolated from 2,965 root samples or 95 per cent of the total number. The fungi associated with the roots were classified into 11 groups. Most of the groups were made up of fungi ordinarily considered nonpathogenic. Nine per cent of the fungi isolated produced only sterile mycelial mats in culture or were otherwise judged unidentifiable. Certain of these unclassified fungi were locally abundant from some trees or areas, but entirely absent from others, regardless of tree condition.

Phytophthora cinnamomi was the only recognized pathogen isolated from shortleaf pine roots. It has been found widely distributed in the soil in littleleaf areas and may be one of the factors contributing to the deterioration of shortleaf pine on certain soils.

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ANTHRACNOSE OF PEACHES[1]

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Although anthracnose of peaches has been reported from a number of places in the United States[3] apparently it has been considered of little importance in the orchard and on the market. However, this disease has been extremely destructive to peaches in Japan. In 1920 Suematsu and Kuwatsuka (5) said: "The damage was so destructive as practically to annihilate the crop in large areas, and indeed, the earliest and most profitable varieties such as the Amsden June and the Alexander were so susceptible that they had to be eradicated."

Anthracnose appeared in commercial shipments of peaches from Georgia and South Carolina during the 1947–1950 seasons and caused appreciable damage to fruit arriving on the Northern markets. It was first observed, in small amounts and in various stages of development, in several carloads of fruit shipped from Georgia in July 1947 (4). The decay ranged up to 12 per cent in a few carloads. In 1948 the disease again occurred in Georgia shipments and in a few car lots from South Carolina. Apparently this disease is becoming more serious, for in 1949 some lots of Georgia peaches were rejected at shipping point because of anthracnose decay. The work reported in this paper was undertaken to determine the identity of the causal organism or organisms, the mode of infection, and the rate of development of fruit decay at different temperatures in the laboratory, in transit, and on the market.

SYMPTOMS

Typical lesions of anthracnose on market peaches usually range from $\frac{1}{4}$ to $\frac{3}{8}$ in. in diameter, but some $\frac{1}{2}$ in. and larger have been noted (Fig. 1, A–C); the depth varies from $\frac{1}{8}$ to $\frac{1}{2}$ in. Some of the larger ones are rather firm, moist, brown decay, in the shape of a cone with the apex extending to the seed. Externally the lesions are brown, firm, and slightly sunken, and have rather definite circular margins. Under humid conditions a fine grayish mycelium can be seen on spots $\frac{1}{4}$ in. in diameter and larger. The larger lesions often have the salmon-pink, sticky spore masses characteristic of anthracnoses on other fruits and vegetables (Fig. 1, D–F).

[1] Investigations conducted by the Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, in cooperation with the Department of Botany at the University of Chicago.

[2] Respectively, Senior Pathologist, Pathologist, and formerly Scientific Aid; Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture.

[3] Collaborators of the Plant Disease Survey have reported anthracnose on peaches from Arkansas, Florida, Georgia, Maryland, New Jersey, Ohio, Tennessee, and Texas.

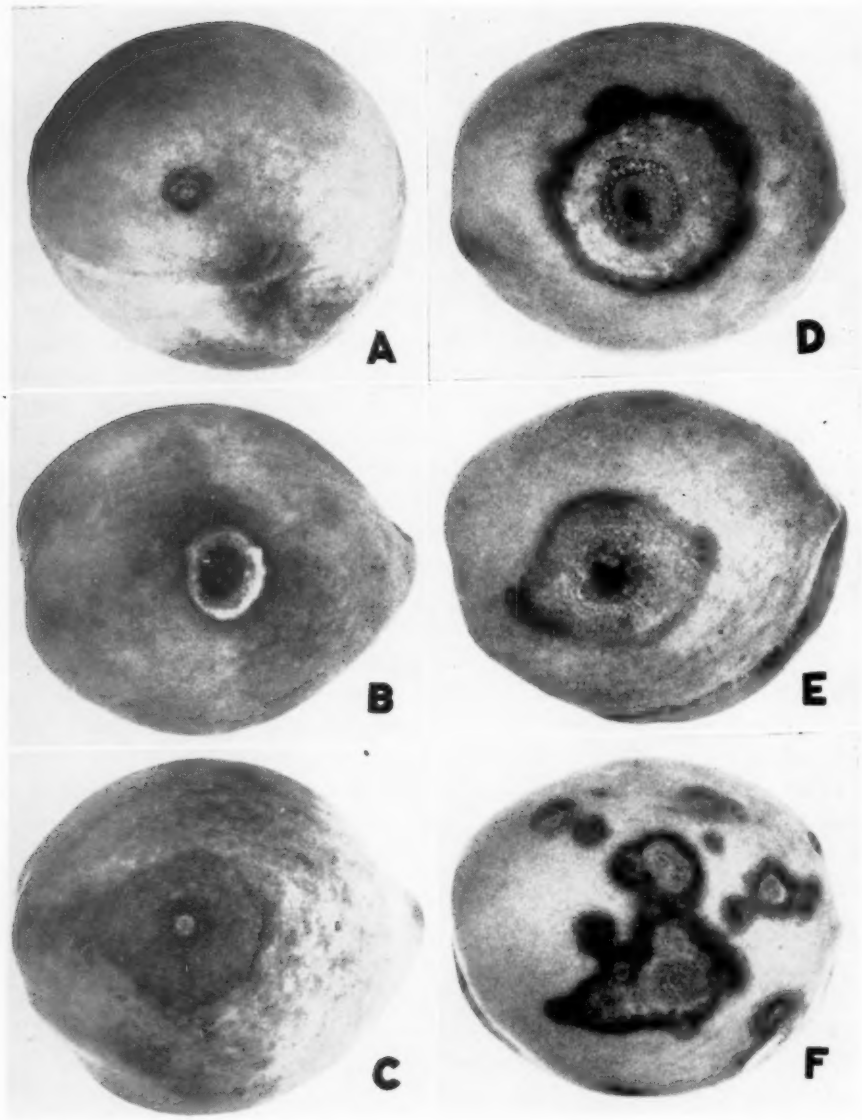

FIG. 1. Peach anthracnose. A, B, C, natural infections; D, E, F, artificial infections. D, wound inoculation; E, mycelium contact inoculation; F, spray inoculation by spores in a water suspension.

THE CAUSAL ORGANISM

Anthracnose lesions on peaches on the market are unusually free from secondary infections. Even in lots of fruit that have a high percentage of brown rot and Rhizopus rot, these decays seldom originate at the anthracnose lesions, although they may eventually overrun the anthracnose lesions. Pure cultures with the characteristic growth and spore measure-

ments of *Glomerella cingulata* (Ston.) Spauld. & Schrenk were obtained from all isolations made from typical anthracnose lesions.

The usual variations among strains as noted by other investigators (1, 2, 6, 7) have been observed in the peach isolates. About 90 per cent of the isolates have been of the grayish-green type, and 10 per cent of the pink type, when cultured on potato-dextrose agar. The conidia of the gray isolates average $16.4 \times 6.0\mu$; those of the pink isolates, $14.8 \times 5.2\mu$. The spores from acervuli of the pink isolates were consistently smaller and more pointed at the ends than those of the gray isolates. Somewhat similar differences between gray and pink isolates from apple were reported by Shear and Wood (7). However, their pink isolates grown on cornmeal agar produced a decided pink color in the medium, whereas the pink isolates from peaches used in the present study produced practically all of the color in the mycelium and spores when grown on potato-dextrose agar and on cornmeal agar. None of the chromogenic isolates from apples studied by Shear and Wood produced perithecia, and none of the pink isolates from peach discussed in this paper tended to produce perithecia. Pink isolates from apples used in comparative studies (Table 1) likewise did not produce perithecia.

Some of the gray isolates from peaches have produced perithecia in pure culture on potato-dextrose agar and on decaying ripe peaches. The perithecia range from 84 to 156μ in diameter, the asci average $65.0 \times 11.4\mu$, and the ascospores $18.6 \times 5.7\mu$.

In addition to the morphological differences between the gray and pink isolates, physiological differences, as shown by growth rate in pure culture and in peach fruit, were also noted. The gray isolates grow more rapidly in culture above 50° F. and cause more rapid decay of peaches at temperatures above 60° F. than do the pink isolates; the pink isolates grow more rapidly in culture below 50° F. and produce more extensive decay in peaches at temperatures below 60° F. than do the gray isolates.

A comparison was made of isolates of *G. cingulata* from peach and apple with *G. gossypii* Edge. from cotton, *G. musarum* Cke. & Mass. from banana, and *Colletotrichum phomoides* (Sacc.) Chester from tomato. Potato-dextrose agar plates were inoculated with small portions of agar containing mycelium from cultures of each of the species. The plates were incubated at 75° F. Diameters of the colonies, considered the indices of growth, were measured at the end of 4 days and characteristics of the colonies noted (Table 1). Although variations in growth rate and colony characteristics occurred in this test, this is not unusual, and it is believed that these growth characteristics, together with morphological studies, indicate that the gray isolates of *G. cingulata* from peaches and apples are similar, as are the pink isolates from the same hosts.

Cross inoculation studies. The pink and gray isolates of *G. cingulata* and one strain each of *G. musarum* and *G. gossypii* were used in cross inoculations on apple, peach, plum, and tomato fruits. After wound inocula-

TABLE 1.—*A comparison of Glomerella cingulata isolates from peach and apple with G. musarum, G. gossypii, and Colletotrichum phomoides when grown on potato-dextrose agar at 75° F. for 4 days*

Host	Culture number	Diameter of colony (mm.)	Colony characteristics
Peach	4124	42	Moderate amount of grayish-green mycelium. No visible acervuli.
Do	4173	52	Abundant cottony, grayish-green mycelium. No visible acervuli.
Do	4172	36	Fine, compact, pink mycelium. Orange-yellow acervuli, spores abundant in center.
Apple	4163[a]	38	Fine, compact, pink mycelium. Orange-yellow acervuli, and spores abundant in center.
Do	4164[a]	36	Dull, grayish, somewhat cottony mycelium. No acervuli.
Do	4165[a]	33	Fine, compact, pink mycelium with some yellow sectors. Yellow acervuli and spores in center.
Do	4196[b]	36	Fine, pink mycelium. Yellow acervuli in center.
Tomato	4166	36	Scanty, grayish mycelium. Abundant black acervuli in center.
Banana	4187	84	Flocculent white to faint pink mycelium. Yellow to brown dotlike acervuli in center.
Cotton	4197[c]	60	Abundant cottony, gray mycelium. Yellow acervuli in center.

[a] Furnished by H. W. Anderson.
[b] Furnished by M. C. Goldsworthy.
[c] Furnished by C. W. Edgerton.

tions were made, the fruits were incubated at 75° F. Lesion diameter was measured at the end of 3 and 5 days. The data appear in table 2.

In other cross inoculation studies with the peach and apple strains of *G. cingulata,* the resulting lesions were similar in character. For example, firm ripe Bartlett pears inoculated with a gray strain from apple (No. 3617) and a gray strain from peach (No. 4124) developed lesions 22 and 20 mm. in diameter, respectively, in 5 days at 72° F. In a later study, *G. cingulata* from magnolia received from Fowler (3), when inoculated into peaches through wounds, developed typical anthracnose lesions 29 mm. in diameter after 5 days' incubation. Weimer and Dunegan (8) recently have shown that *G. cingulata* from blue lupine is also pathogenic to peaches.

TABLE 2.—*Results of cross inoculations in wounds when incubated at 75° F.*

Culture	Original host	Incubation period (days)	Lesion diameter, in mm.			
			Apple	Peach	Plum	Tomato
G. cingulata 4172 (pink isolate)	Peach	3	8	9	6	10
		5	13	15	19	19
G. cingulata 4173 (gray isolate)	Do	3	12	13	13	14
		5	18	21	24	25
G. musarum 4187	Banana	3	0	0	0	0
		5	0	0	0	0
G. gosspyii 4197	Cotton	3	6	11	0	14
		5	11	14	0	26

These studies indicate that the source of inoculum for peaches in the southern States may be apples, lupine, magnolia, other peaches, or possibly other host plants of *G. cingulata* in the vicinity. Although *G. gossypii* will produce decay in peaches, apples, and tomatoes when inoculations are made through wounds, natural infection of peaches from cotton plants affected with anthracnose has not been reported. The banana anthracnose organism was unable to rot any of the fruits tested.

TEMPERATURE STUDIES

Vigorously growing pure cultures of typical gray and pink isolates of *G. cingulata* from peaches were plated on potato-dextrose agar in Petri dishes and incubated, at 5-degree intervals from 40° to 95° F. The average diameter of 10 or more colonies after 7 days' growth of each culture at the various temperatures is shown in figure 2. No colonies

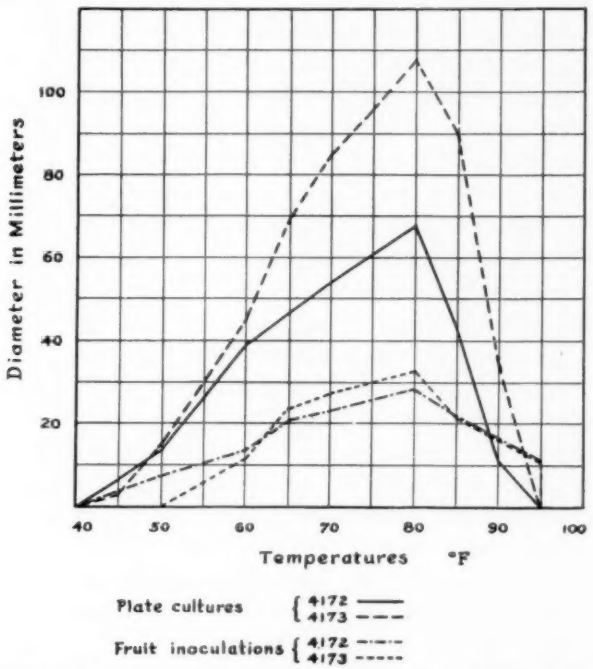

FIG. 2. Influence of temperature on typical gray (No. 4173) and pink (No. 4172) isolates of *Glomerella cingulata* from peaches, when grown in pure culture on potato-dextrose agar plates and in wound-inoculated peaches, for 7 days.

of either culture developed within one week at 40° or at 95° F. Each organism, however, remained alive in each culture plate and began to grow when the plate was removed to room temperature. Optimum growth occurred at 80° F. for each isolate. In numerous trials the colony diameter for the pink isolate (4172) was consistently greater than for the gray isolate up to about 50° F., whereas at higher temperatures the gray isolate (4173) produced the larger colonies.

The influence of temperature upon the rate of decay development was determined by inoculating firm-ripe peaches in slight scalpel wounds with pure cultures of the same pink and gray isolates of *G. cingulata* used in the plate-culture studies. The average diameter of 10 or more lesions resulting from such inoculations is shown for each temperature in figure 2.

As in the case of plate cultures, the growth of each isolate was optimum at 80° F. A marked difference in development of decay occurred between 40° and 60° F. The pink isolate (4172) invaded the peach tissue at slightly above 40° F., whereas the gray isolate (4173) produced no decay unless the temperature was above 50° F. At 60° F. the two isolates produced lesions of about the same size, but between 65° and 85° F. the gray isolate consistently produced the larger lesions.

Both the pink and gray cultures of *G. cingulata*, applied as mycelium or spores and without wounding, were pathogenic to hard-ripe and firm-ripe peaches that had no visible injuries. Figure 1, D–F, shows the results obtained with three methods of inoculation after 7 days at 80°–85° F. As might be expected, the wound inoculations (D) produced the largest lesions; those from contact inoculations by mycelium (E) were slightly smaller, and the smallest were from inoculations made by atomizing with a water suspension of spores (F).

Tests were made to determine possible differences between the pink and gray isolates in percentage of spore germination at 50° F., a temperature often found in transit refrigeration for peaches, and at 80° F., the optimum temperature for growth of these isolates. Conidia from 14-day-old cultures were suspended in Czapek's solution, care being exercised to avoid seeding the solution too heavily. Four drops of the spore suspension from each of the respective cultures were added to each of two slides, which were placed in a saturated atmosphere at 50° and 80° F., respectively, for 20 hr. At 50° F., 78 per cent of the spores of the pink isolate germinated with long germ tubes, whereas only 31 per cent of those of the gray isolate germinated, and the germ tubes were short. At 80° F., 89 per cent of the spores of the pink isolate germinated and 92 per cent of those of the gray isolate germinated. Thus there were no significant differences in the percentage of germination at 80° F., but at 50° F. more than twice as many spores of the pink isolate than of the gray isolate germinated. This may explain in part the more rapid growth of the pink isolate on culture media and in inoculated peaches at low temperatures.

DEVELOPMENT OF ANTHRACNOSE OF PEACHES IN TRANSIT AND IN HOLDING AT ROOM TEMPERATURES

Some of the anthracnose lesions that develop on peaches on the tree are large enough to be seen and graded out at the packing shed, but undoubtedly some small lesions are overlooked. These small lesions and others resulting from infection of wounds and from contamination of apparently healthy fruit with spores during harvesting, grading, and packing of the fruit for shipment cause the decay during transit and marketing.

In order to obtain data on the development of anthracnose decay during transit and ripening on the market, one bushel of peaches was carefully selected for freedom from decay and blemishes, and the peaches were inoculated with a pure culture of *Glomerella cingulata* through slight scalpel wounds. Fruit in another bushel was inoculated by atomizing with a water suspension of spores from a pure culture of this fungus. Two bushels of peaches from the same lot, one injured with sterile scalpel punctures, and one sprayed with tap water, served as controls. These four lots were included in an ordinary refrigerated commercial carload of peaches shipped from Inman, S. C., to Chicago, Ill., in August 1948. A thermometer placed in one of the baskets of fruit recorded 90° F. when the fruit was loaded; 65° F. after one day in transit; 55° F. the second day, and 48° F. the third day and on delivery.

On arrival in Chicago after 3 days in transit all of the wound-inoculated fruit showed anthracnose lesions averaging ½ in. in diameter. One naturally infected fruit was also found in this lot. All of this fruit was repacked and held at retail store temperature (80°–85° F.) for 3 days. On reinspection the wound-inoculated fruit showed lesions ranging from ½ to 1 in. in diameter. Eight contact infections had occurred during this time and four natural infections ⅛ to ¼ in. in diameter were found. The controls remained free of the disease.

The peaches atomized with a water suspension of spores showed 8.5 per cent infection on arrival, the lesions ranging from 1/16 to ⅛ in. in diameter; after 3 days at 80°–85° F., 33.5 per cent showed anthracnose. The control basket contained one fruit with natural infection by *Glomerella*.

The high percentage of successful wound inoculations was not surprising in view of the success with this type of inoculation in several laboratory tests by the authors as well as by other investigators. The spray inoculations with a water suspension of spores, however, resulted in more infections than had been anticipated considering the very hot, dry condition of the fruit when inoculated. The slight amount of moisture contributed by the atomizer at the time of inoculation, in addition to the humidity of the pack in the refrigerator car, apparently was sufficient to allow spore germination and subsequent invasion of the fruit in the absence of any visible wounds. This would indicate that severe anthracnose might develop in shipments of apparently healthy fruit during seasons when an abundance of spores in the orchards may contaminate fruit during harvesting and packing.

During the season of 1949 somewhat similar inoculation tests were made on four half-bushel baskets of commercially brushed fruit, packed in the usual manner and shipped by express without refrigeration from Spartanburg, S. C., taking 3 days in transit. The peaches were carefully selected for freedom from decay and blemishes. Those from one basket (lot 1) were dipped in a spore suspension of *G. cingulata*, and for a control, those in the second basket (lot 2) were dipped in tap water. Peaches in the

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third basket (lot 3) were lightly stabbed with the point of a fine dissecting needle that had been dipped in a water suspension of spores, and those in the fourth basket (lot 4) were stabbed with a sterile needle, as a control. On arrival in Chicago the fruit in lot 1 had 43 per cent anthracnose, with lesions $\frac{1}{8}$ to $\frac{1}{4}$ in. in diameter and larger decayed areas where lesions had coalesced. On reinspection after 3 more days at 80° F. these peaches were so badly infected with brown rot and Rhizopus rot that no accurate data could be obtained on the further development of the anthracnose lesions. The control peaches (lot 2) had no anthracnose on arrival but after another 3 days at 80° F. 1.7 per cent of the fruit showed natural infections.

The peaches in lot 3 had 79 per cent infection on arrival, the anthracnose lesions ranging from $\frac{1}{4}$ to $\frac{1}{2}$ in. in diameter. The fruit was held for 3 days at 80° F., and then had 85 per cent infection with lesions ranging from $\frac{3}{8}$ to $\frac{3}{4}$ in. in diameter. The control fruit (lot 4) had no anthracnose on arrival or after being held 3 days at 80° F.

These experiments show that moist fruit contaminated with spores, or fruit with slight wounds made during harvesting and packing, may develop a high percentage of anthracnose rot during transit and marketing in seasons when there is an abundance of spores contaminating the fruit at harvest time.

SUMMARY

Peach anthracnose was recognized as an important disease of Georgia-grown peaches on the market in 1947. Since that time the disease has been found in increasing amounts in orchards in Georgia and South Carolina and in shipments from these States.

Isolations from typical lesions yielded pure cultures of *Glomerella cingulata*. Most cultures were of the gray type, but many of the pink type were also obtained. The gray isolates produced the *Glomerella* stage in pure culture and in inoculated peaches. The pink isolates never developed the *Glomerella* stage. The gray isolates grew more rapidly in culture above 50° F. and caused more rapid decay of peaches above 60° F. than did the pink isolates. The pink isolates grew more rapidly in culture below 50° F. and produced more extensive decay in peaches below 60° F. than did the gray isolates. The optimum temperature for both types of isolates is 80° F. as indicated by growth in culture and in inoculated fruit.

Hard-ripe and firm-ripe peaches are readily infected by inoculation through wounds, by mycelium in contact with uninjured tissue, and by spraying of fruit with a water suspension of spores.

Transportation tests with South Carolina peaches under standard refrigeration show that fruit inoculated in wounds may develop lesions $\frac{1}{2}$ in. in diameter in 3 days. Under the same conditions, fruit sprayed with a water suspension of spores may develop lesions 1/16 to $\frac{1}{8}$ in. in diameter.

All transportation tests indicate that there is danger of serious development of anthracnose in peaches through wounds made during harvesting and

packing, and by contamination with spores in seasons when there is an abundance of inoculum in the orchards.

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INSECT TRANSMISSION OF ALFALFA WITCH'S-BROOM VIRUS TO OTHER LEGUMES[1]

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Transmission of the alfalfa witch's-broom virus by means of the leafhopper *Platymoideus acutus* Say. was reported in 1946 by the junior author, who also showed by grafting tests that *Medicago lupulina* L. and *M. hispida* Gaert. were susceptible to infection.[3] The insect vector, now designated as *Scaphytopius* (*Cloanthanus*) *dubius* (Van Duzee),[4] has been used in recent transmission tests which have extended the list of symptom-expressing hosts to include 13 other species of legumes.

Twenty-three species or varieties of forage legumes[5] were inoculated with the virus by means of viruliferous leafhoppers. Groups of infective insects, from one to 20 in number, were caged on a series of healthy potted plants, at least one plant in each series receiving 10 or more insects. Feeding periods on the test plants varied from 1 to 35 days with at least one inoculation period in each series lasting 7 days. Following inoculation, the plants were sprayed with nicotine sulphate to kill the insects, and grown in the greenhouse during the observation period. Production of symptoms typical of those on alfalfa was considered sufficient evidence of infection. Noninoculated checks were maintained in all cases.

As shown in table 1, fifteen species expressed typical witch's-broom symptoms following inoculation. These represent the genera *Medicago*, *Melilotus*, *Trifolium*, *Lotus*, *Astragalus*, *Lathyrus*, and *Hedysarum*.

Of the eight species giving negative results, four were representatives of the above genera, suggesting that continued testing might demonstrate their susceptibility also. Both species of *Onobrychis* in ten separate tests failed to show symptoms of witch's broom.

Symptoms on all infected plants were striking and typical of witch's-broom symptoms on alfalfa. Figure 1 illustrates the dwarfing and proliferation of stems that occurred on representative species. Symptom expression on *Trifolium pratense*, *T. repens*, and *Medicago lupulina* was typical of

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[3] Menzies, J. D., Witches' Broom of Alfalfa in North America. Phytopath. 36: 762–774. 1946.

[4] Specimens of the leafhopper used by Menzies were identified by R. H. Beamer, University of Kansas, as *Platymoideus acutus* Say. This genus is no longer considered valid, the species now being assigned to the genus *Scaphytopius*. Further study of this insect by P. W. Oman, Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, and by D. M. DeLong, Ohio State University, has shown the species to be *Scaphytopius* (*Cloanthanus*) *dubius* (Van Duzee). L. W. Hepner, Hays College, Kansas, considers *dubius* to be a variety of the species *acutus*.

[5] Most of these were obtained through the courtesy of John L. Schwendiman, Manager, Pullman Nursery Unit, Soil Conservation Service, U. S. Department of Agriculture.

TABLE 1.—*Results of insect-transmission tests with alfalfa witch's-broom virus on other legumes*

Species tested (positive results)	No. of plants		Species tested (negative results)	No. of plants	
	Inoculated	Infected		Inoculated	Infected
Medicago lupulina L.	5	5	*Medicago lacinata* Mill.	5	0
M. hispida Gaert.	5	1	*Astragalus cicer* L.	1	0
M. falcata L.	20	3	*A. canadensis* L.	1	0
M. ruthenica Trauty.	5	1	*Vicia variabilis* Freyn & Sint.	3	0
Melilotus alba Desr.	5	5	*Lathyrus pratensis* L.	1	0
Trifolium pratense L.	5	5	*Onobrychis viciafolia* Scop.	5	0
T. repens L. (white clover)	5	4	*O. transcaucasica* Gross.	5	0
T. repens L. (Ladino clover)	5	4	*Swainsona salsula* (Pall.) Taub.	2	0
T. subterraneum L.	5	4			
Lotus corniculatus L.	5	3			
Astragalus falcatus Lam.	4	1			
A. chinensis L.	7	2			
A. mortoni Nutt.	4	1			
Lathyrus latifolius L.	4	1			
Hedysarum coronarium L.	2	1			

that observed in the field on these species. Witch's-broom symptoms in *Melilotus* sp. have been noted previously in Alberta[6] but have not been found naturally occurring in Washington.

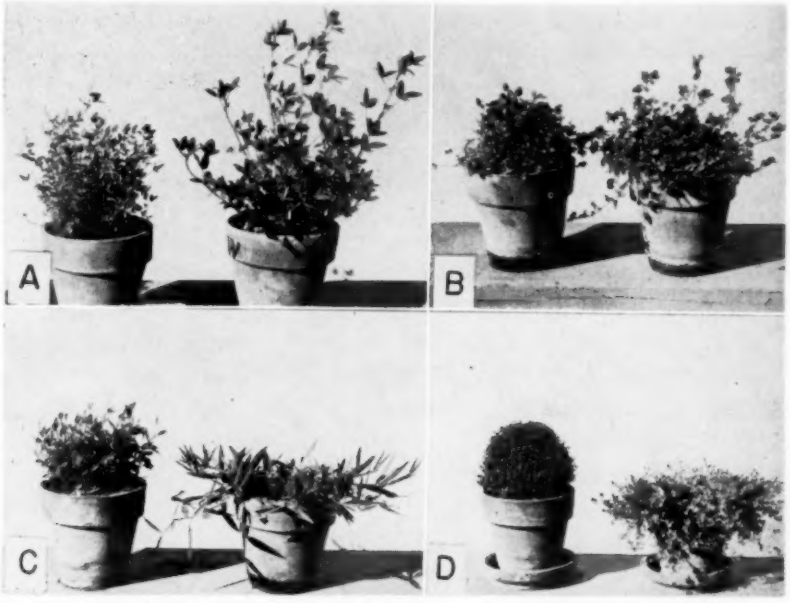

FIG. 1. Symptoms induced in various legumes by leafhopper inoculation with the alfalfa witch's broom virus. (Plants were photographed approximately 18 months after inoculation.) The plant on the right in each case is healthy. A, *Melilotus alba;* B, *Medicago lupulina;* C, *Lathyrus latifolius;* D, *Lotus corniculatus.*

[6] Cormack, M. W. (Witches' Broom on Alfalfa and Sweet Clover.) *In* Ann. Rpt. Canadian Plant Dis. Sur. 20: 20 and 22. 1941.

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The incubation period of the disease in the plants following insect inoculation was prolonged. The minimum interval prior to recognizable symptoms was 74 days. This is in agreement with the results reported with graft inoculation. Infections were obtained with previously noninfective insects which fed for 3 days on virus-infected alfalfa and 7 days on healthy *Lathyrus latifolius*, indicating a latent period, if any, of less than 10 days. The virus was transmitted in three cases during a 24-hr. feeding period on the test species. One group of 20 insects which had been reared on infected alfalfa remained infective for 13 days, during which they inoculated five sweet-clover plants in serial transfers. Other retention periods of 9, 10, and 11 days were noted. More extended periods were not tried.

Because of the long incubation period of the virus it has not been feasible to test the susceptibility of annual legumes. These, if susceptible, probably mature before showing recognizable witch's-broom symptoms. Only one attempt has been made to test host range outside of the Leguminosae. This was with potato (*Solanum tuberosum* L.), a crop on which a witch's-broom disease of similar type occurs in the Northwest. Although the witch's-broom vector is found occasionally on potatoes, in cage experiments it did not survive more than 3 days. Five trials were made in which a population of ten living, infective leafhoppers was maintained on healthy potato plants by necessary additions of insects for 13 days. No symptoms of witch's broom occurred on these potatoes, and all tubers from them subsequently produced normal plants.

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SOME NUCLEAR PHENOMENA IN PHYSALOSPORA OBTUSA[1]

ROBERT AYCOCK[2]

(Accepted for publication January 15, 1951)

Physalospora obtusa (Schw.) Cooke causes a rot of apple fruits and a necrotic spotting of apple leaves. Both of these diseases occur throughout the eastern United States and are of considerable economic significance in commercial apple production.

Prior to 1913, studies on the causal fungus were concerned with the imperfect stage, *Sphaeropsis malorum* Peck. In that year, however, the relationship of the conidial and ascogenous forms of the fungus was reported by Hesler (6). Although the existence of the perfect stage was thus established, its potentialities as a source of variation and its significance in the epidemiology of frogeye leaf spot and black rot have been neither fully explored nor evaluated. Moreover, reports of the occurrence of the perithecial stage since that time have shown certain inconsistencies. Hesler (7) reported that the perfect stage occurred only rarely and Lyle (12) was unable to find perithecia in North Carolina orchards during the period 1941 to 1943. These reports lend credence to the general assumption that pycnidia overwintering in the bark of dead apple branches constitute the principal source of inoculum for primary leaf infections in the spring. Crabill (1) attributed both fruit and leaf infections throughout the season to conidia, and Hesler (7), and Lyle (12) considered perithecia to be of little importance in the epidemiology of frogeye leaf spot and black rot.

However, Shear, Stevens, and Wilcox (15) and Stevens (16), in extensive mycological collections, found perithecia of *Physalospora obtusa* occurring abundantly in the bark of dead twigs of many species of woody plants. Oftentimes, associated with these fruiting bodies they also found perithecia of a morphologically similar species, *Botryosphaeria ribis* G. & Dug.

Since 1946 numerous collections have revealed that perithecia of *Physalospora obtusa* occur abundantly in the bark of dead apple branches in North Carolina orchards. Moreover, periods of heavy ascospore discharge have been found to precede primary infections of apple leaves in the spring. These findings emphasized the need for a critical reinvestigation of the life history of the causal organism, and especially for a careful study of the development of the perfect stage. The present report is concerned prin-

[1] Condensed from a portion of a thesis submitted to the Graduate Faculty of the North Carolina State College of Agriculture and Engineering, University of North Carolina, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1949. Published with the approval of the Director as Paper No. 365 of the Journal Series of the North Carolina Agricultural Experiment Station.

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cipally with nuclear phenomena associated with perithecial and pycnidial development.

MATERIALS AND METHODS

Perithecial material used in these studies was obtained from orchards near Boomer, North Carolina, and near Raleigh, North Carolina.

Several fixatives were employed, including La Cour's 2BE, Flemming's Medium, Benda's, formalin-acetic-alcohol, Bouin's, and Karpechenko's and Randolph's modification of Navashin fluid (8). The osmic fixatives were generally unsatisfactory, however, and best results were obtained with the two Navashin-type fluids and formalin-acetic-alcohol.

Material for fixation was prepared by removing the bark from twigs or branches and cutting it into small sections about 6×8 mm. Most of the material was killed and fixed immediately after collection, although in some instances fixation was delayed until the material had been incubated in moist chambers for varying periods. Paraffin infiltration was accomplished by means of the tertiary butyl alcohol technique. Slides were prepared from the embedded perithecia by cutting microtome sections 4–6 μ thick and staining with Heidenhain's iron alum haematoxylin.

OBSERVATIONS

Asci: early stages. Young perithecia are filled with closely packed delicate hyphae. The young asci arise as minute budlike protrusions from the base of the fruiting body and when first recognizable average about 5×15 μ. Crozier formation and nuclear fusion to form the primary ascus nucleus were not observed with certainty, although structures resembling binucleate penultimate cells were seen in a few cases. The primary ascus nucleus in the earliest stages of development measures about 2.8–3.5 μ in diameter.

As the young asci push upward into the cavity of the perithecium, the closely packed, delicate hyphae spread apart and appear as indistinct, anastomosing hyphal segments. No true paraphyses were observed.

At the beginning of the first nuclear division, the asci measure about $10.5–14 \times 28–40$ μ. The prophase nucleus ranges from 6.5 to 8.4 μ in diameter and is bordered, usually above and below, by a highly vacuolate region (Fig. 1, A). The nucleolus is at first very conspicuous averaging 1.5 μ in diameter. A distinct nuclear network is present and consists of thin threads which wind and coil through the nuclear cavity. Beaded strands, suggestive of chiasmata, are clearly visible (Fig. 1, A). Central bodies and radiating spindle fibers as reported in other fungi were not evident.

Chromosomes were clearly visible at diakinesis (Fig. 1, B). At this stage 10 chromosome pairs appeared to be present and definite pairing was noted in as many as six of these. Two long chromosome pairs (Fig. 1, B) measuring about 2.5 μ in length were observed in a number of preparations. In most of the first-division figures, only seven or eight recognizable chromo-

some elements were present, probably because full chromosome complements were not present in the sections. In no case, however, were more than 10 bivalents observed. At diakinesis only a suggestion of the nuclear membrane remains, and the nucleolus has disappeared. The chromosomes

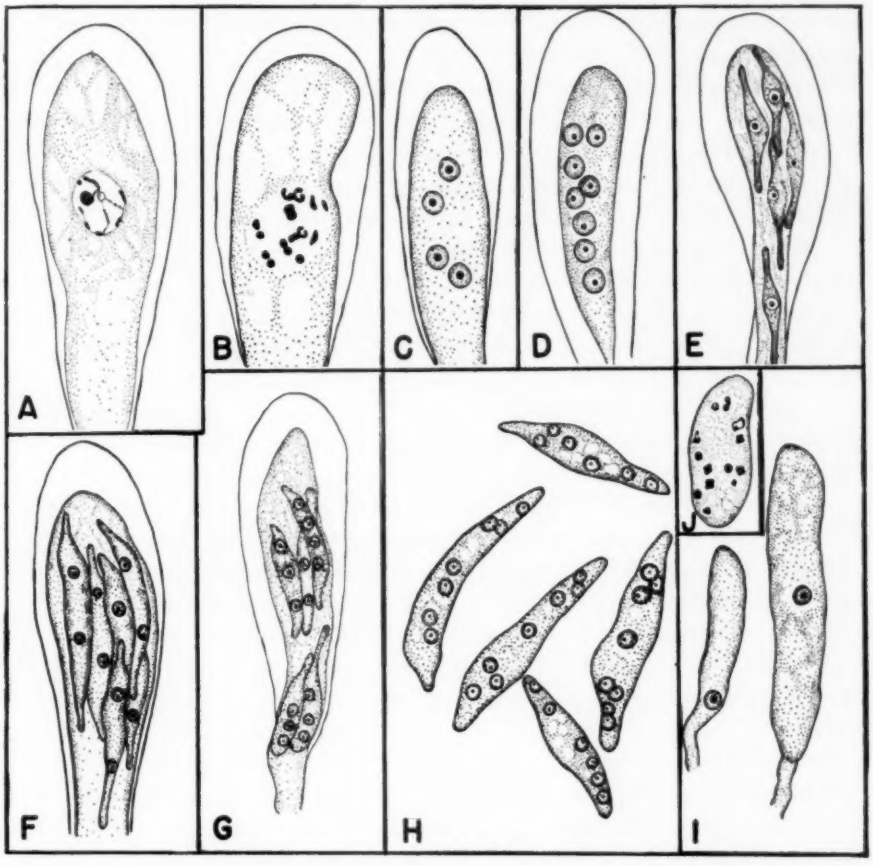

FIG. 1. Some nuclear details in developing asci and conidia of *Physalospora obtusa*. A. First division prophase showing conspicuous nucleolus and chromosomes (×1,400). B. Diakinesis. The nucleolus has disappeared. Six bivalents are evident. Pairing is not visible in the other four (×1,400). C. Young ascus at the four-nucleate stage (×1,100). D. Eight uninucleate ascospores immediately following the third division (×1,100). E. Section of an ascus showing five uninucleate ascospores shortly after elongation has begun (×1,100). F. Binucleate, elongate ascospores (×1,100). G. Four-nucleate ascospores (×800). H. Six-, seven-, and eight-nucleate ascospores. Spores at this stage are still immature (×1,100). I. Early stages in conidial formation (×1,400). J. Hyaline, multinucleate conidium (×500).

are scattered throughout the nuclear cavity and the adjacent cytoplasm continues to be highly vacuolate.

The position of the nuclei at the binucleate stage indicates that the plane in which the first division occurs is in the general direction of the longitudinal axis of the ascus, although the plane is usually somewhat oblique.

No well-defined figures of the second division were noted. Loosely ar-

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ranged chromatin masses suggestive of telophase I and prophase II were seen, but it was not possible to distinguish individual chromosomes. Whether the rarity of second-division figures was due to the rapidity with which this division occurs as reported in other species (3, 11) is not known.

The position of the nuclei in the four-nucleate stage (Fig. 1, C) indicates that the second-division figures lie in a diagonal or oblique plane. Asci at this point measure about 10–12 × 40–45 μ.

Third-division figures are fairly abundant, which may indicate that the nuclei go into a brief resting stage at the conclusion of the second division. Chromosomes as individual entities were rarely distinguishable after meiosis I, and with the techniques employed they usually appeared as aggregates of chromatin in the third division. Both transverse and oblique planes were observed in the dividing nuclei. At this stage of development, the chromatin material lies in at least three planes of focus, which suggests that both polar and longitudinal views are obtained. This distribution of the nuclei, however, is consistent with the biseriate arrangement of the ascospores in the mature ascus.

Ascospore delimitation and maturation. Eight spherical ascospores measuring 2.5–3.5 μ in diameter are delimited at the conclusion of the third nuclear division. Astral rays functioning in the process of delimitation were not observed and the mechanism of this process was not determined. Only a faint membrane was seen in the nucleus of the young spore. A deeply staining chromatin mass was present, however, and the average diameter of the nuclei was about 2.0 μ (Fig. 1, D). The diameters of young spores do not greatly exceed those of nuclei in the four-nucleate ascus (Fig. 1, C), but the presence of definite cleavage furrows in the cytoplasm suggests very strongly that the young spores are actually cells and not free nuclei.

The young ascospores now go through an unusually striking process of elongation and growth until the size and more or less ellipsoidal shape of the mature ascospores are attained (Fig. 1, D–H). Early elongation occurs quite rapidly until the slender, filiform spores measure some 18 to 20 μ in length. Up to this point the ascospores remain uninucleate. Subsequently, however, several successive nuclear divisions occur, so that the mature spore may contain as many as 16 to 20 nuclei. The spores are still very slender at the binucleate stage (Fig. 1, F), but by the time the eight-nucleate stage is reached some indication of the final shape of the mature spore is apparent.

Conidia. A study of paraffin sections of pycnidia produced in pure culture on potato-dextrose and oatmeal agars from monoascosporic isolates of *Physalospora obtusa* revealed that conidia in their earliest stage of development are uninucleate. One nucleus passes from the conidiophore into the developing spore and the uninucleate condition is maintained until a septum is laid down at the base of the conidium (Fig. 1, I). Subsequently a number of nuclear divisions occur, so that later the conidia become multinucleate

(Fig. 1, J). The nuclear condition was determined only in hyaline conidia. It was not possible to study nuclear phenomena in mature conidia because the brown sheath which envelops them apparently interferes with the penetration of fixatives and stains. There is no reason, however, to suppose that the multinucleate condition is not maintained in these mature conidia.

Examinations of the mycelia in these same pycnidial preparations showed that hyphal cells contained at least two to five nuclei, although the staining results obtained in these instances were less satisfactory than those obtained with any other material.

DISCUSSION

Certain cytological details of perithecial development in *Physalospora obtusa* are quite different from those reported for other Ascomycetes. Perhaps the most significant difference is found in the sequence of events associated with maturation of the ascospores. While the spores are spherical to elliptical when first delimited, they elongate until they are actually filiform in shape. In unstained preparations, they appear as long intertwining strands. Subsequently, they also increase in width, until the ellipsoidal to inequilateral shape of the mature ascospore is attained.

Faull (2) has shown that in *Laboulbenia chaetophora* Thaxter a process of elongation occurs in the young spherical ascospores, and Lewis (10) also indicates that the elongate ascospores of *Pleurage zygospora* (Speg.) Kuntze are originally round. Jones (9), on the other hand, reports that in *Rhytisma acerinum* (Pers.) Fries, well-defined planes of cleavage occur immediately following the third division, the direction of which indicates that they are elongate when cut out. However, no account of the maturation of ascospores through three successive stages of development as described herein for *Physalospora obtusa* has come to the writer's attention.

The fact that ascospores, conidia, and hyphal cells are multinucleate is of considerable importance in furnishing a basis for genetic studies of the organism. The possibility of variation due to heterokaryosis cannot be completely excluded, in spite of the fact that both ascospores and conidia are apparently homokaryotic when first formed. It is conceivable that fungi with multinucleate hyphal cells are potentially more variable than those which consist of uninucleate cells, since a single mutation in a single nucleus would render the cell in question heterokaryotic. In fact, evidence that multinucleate fungi are highly variable has been presented in recent years (4, 5, 14).

Mohendra and Mitra (13) have demonstrated that single conidia of *Physalospora obtusa*, when isolated from the same pycnidium, sometimes give rise to different types of mycelia. They postulated that saltants had arisen, but that instead of sectoring, they intermingled and cooperated in pycnidial production, conidia of each of the representative strains being produced. These same results, however, could be explained by the segregation of genetically different types of nuclei into the young uninucleate conidia. It is not unlikely that both saltation and heterokaryosis are re-

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sponsible for the many variable forms of *Physalospora obtusa* which occur in pure culture.

An extension of the present study, especially along certain lines, is indicated. The abundance of perithecia of *Physalospora obtusa* in North Carolina orchards points out the possibility that hybridization and segregation may account in part for the variability of this species. Until knowledge related to sexuality in the organism is forthcoming, however, the importance of perithecia as a source of genetic variation can only be speculated upon.

The abundance of perithecia, also, suggests the interesting and significant possibility that the ascosporic and conidial stages may play complementary roles in the life history of the organism. Moreover, studies on the relative abundance of these two stages in orchards throughout the year should give some indication of the importance of perithecia in the epidemiology of black rot and frogeye leaf spot.

SUMMARY

Perithecia of *Physalospora obtusa* occur abundantly in the bark of dead apple branches in North Carolina.

The chromosome number in the primary ascus nucleus is probably n = 10. Three successive nuclear divisions occur in the young ascus which result in the formation of eight free nuclei.

An unusual type of ascospore maturation follows the third nuclear division. The young ascospores are spherical when first delimited, but soon undergo rapid elongation, no increase in width occurring until they measure about $3 \times 20\ \mu$. Coincidentally with the process of elongation and increase in width, several successive nuclear divisions occur, so that the mature spores are multinucleate.

The conidia when first cut off from the conidiophores are uninucleate, but become multinucleate at maturity. Hyphal cells of the mycelium have also been shown to contain more than one nucleus.

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FIELD EVALUATION OF GLYOXALIDINE COMPOUNDS FOR CONTROL OF CHERRY LEAF SPOT

W. D. Mills and J. D. Van Geluwe[1]

(Accepted for publication February 13, 1951)

The use of low soluble coppers in New York was stimulated by Hamilton who reported in 1938 (1) and 1939 (2) that Cupro K was the safest copper compound available for use on sour cherry and when used with lime and oil-type spreader-sticker was one of the best substitutes for Bordeaux mixture in New York State. Young, in 1939 (8), found less reduction in leaf area below the checks with Cupro K than with any fungicide tested. Murphy (7) in 1940 found significantly higher photosynthesis of sour-cherry leaves with Cupro K than with Coposil, Bordeaux mixture, or lime sulfur.

A cooperative experiment was made in Wayne County, New York, in 1941–1944 inclusive by the senior author and others (5, 6) to determine the effects of four fungicidal programs on Montmorency cherry tree growth, yield and quality of cherries, in this State. Bordeaux mixture 2:6:100 caused severe dwarfing of fruit the first year and was replaced by a $1\frac{1}{2}$:6:100 formula, but the dwarfing continued. Lime sulfur 1:40 also caused severe fruit dwarfing, and leaf-spot control was inferior to that of the copper schedules. Flotation paste, plus an oil sticker, gave poorer leaf-spot control than did the lime sulfur or the two copper schedules. Fruit yield was highest for the trees receiving Cupro K and lowest on the Bordeaux-sprayed trees. Increase in trunk girth was significantly less (1 per cent) on lime-sulfur-sprayed trees than with the other three schedules. The sugar content of Bordeaux-sprayed cherries was highest, and for flotation-sulfur-sprayed trees, the lowest. Size and sugar-content data were taken from 40 trees per treatment. Trunk girth and fruit yields were taken from all trees not showing symptoms of virus yellows or ring spot during the 4-year test, so that these data were based on more than an acre of trees for each schedule.

In 1943 rains at petal-fall stage delayed the first spray by the orchard foreman on the test until the shucks were splitting and heavy leaf-spot infection had occurred. More than half the leaves had lesions when the entire block was sprayed from the ground with Cupro K on July 10. This spray effectively checked the leaf spot without defoliating the trees. The Spray Information Service in New York has regularly recommended such a July eradicant spray of low-soluble copper in years when, for any reason, growers have failed to control leaf spot by the early sprays. During the early 1940's most commercial sour-cherry growers shifted to low-soluble copper

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sprays in part or all of their schedule, and a considerable proportion of them continue to use a low-soluble copper as their standard treatment.

Severe injury to fruit of Montmorency sour cherry in the form of a black ring at the stem end of the fruit followed the use of low-soluble copper sprays in western New York in 1945. From a trace to 55 per cent of the fruit was affected. This stem-end injury occurs more frequently in southern Pennsylvania (3), where most of the injury was caused by low-soluble copper in the shuck spray. Very little injury was caused by copper in the petal-fall spray or the spray following the shuck spray, about 4 weeks later, in the Pennsylvania schedule. In New York in 1945 several of the orchards in which the stem-end injury was most severe received low-soluble copper for the first time in the season in the first fruit-fly spray, applied about 2 weeks after the shuck spray.

Occurrence of this severe injury stimulated interest in other effective fungicides which would not cause fruit injury on sour cherry. A new chemical, 2-heptadecyl-glyoxalidine, reported by Lewis and Groves (3) to be an effective fungicide for the control of cherry leaf spot, was selected for further testing in New York State. The designation "341," assigned to this glyoxalidine compound by the Carbide and Carbon Chemicals Corporation, will be used throughout this paper.

In 1946 cooperative grower-sprayed tests of a slurry form of 341 were set up in four western New York counties. A fifth test in the Hudson Valley was dropped because of wet grounds which prevented the spray applications. Similar tests were continued through 1947 to 1949. Orchards in which there was stem-end injury in 1945 and orchards low in vigor, considered most susceptible to copper injury, were selected; but no stem-end injury occurred during these four years.

Table 1 is a summary of the results in the four western New York counties in 1946. All plots received the five regular sprays recommended in New York, the petal-fall, shuck-fall, first and second fruit fly sprays, and a post-harvest spray. In all four counties 341 slurry was applied on the 341 plots in the petal-fall, shuck-fall, and first fruit fly spray, and Cupro K was used in the second fruit fly and post-harvest sprays. The Cupro K plots received Cupro K in all five sprays. In Wayne County additional plots received 341 in all five sprays.

In all four tests 341 gave better leaf-spot control than the fixed copper. In July the difference in leaf spot was highly significant (1 per cent level) in three orchards and significant (5 per cent level) in the fourth orchard. Less leaf spot was present in October in the trees receiving 341 slurry in the first three sprays than in those receiving five copper sprays, but the difference was not significant. No significant difference in cherry size, as shown by the number of cherries per pound, appeared. Refractometer readings showed no significant difference in sugar content. Fruit defects in two orchards, counted with the aid of a New York State inspector, did

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TABLE 1.—*Cooperative tests of 341 slurry in New York State in 1946*

County	Fungicide[a]	Leaf spot, July 7–11[b]	Cherries per lb.	Solids in solution	Defects
		Per cent	*Number*	*Per cent*	*Number*
Niagara	341	11.1*	109	15.8	8.5
	Cupro K	16.8	114	16.5	10.4
Wayne	341 (5)	13.4**	87	.....	7.0
	341 (3)	13.6**	92	15.1	7.7
	Cupro K	27.2	92	15.2	9.0
Orleans	341	25.9**	123	18.8	.....
	Cupro K	46.9	126	18.8	.....
Monroe	341	33.9**	120	14.2	.....
	Cupro K	60.4	117	13.8	.....
Average	341	21.1**	110	16.0	.....
	Cupro K	37.9	112	16.1	.....

[a] One gal. of 341 slurry, containing 1.25 lb. 2-heptadecyl-glyoxalidine, was used in 100 gal. spray for each application.
[b] * Significant (5 per cent level); ** highly significant (1 per cent level).

not differ significantly in the fruit sprayed with 341 slurry and that sprayed with copper.

In 1946 the slurry form of 341 hydrolized rapidly and this hydrolized form, in a number of tests in New York and in other States, was ineffective in the control of apple scab although effective in leaf-spot control. The general manufacture of the 341 slurry was discontinued in 1947 but a small lot was prepared to repeat the spray experiment with the same materials on the same trees in Niagara, Orleans, and Monroe counties. The results are in table 2.

The Wayne County test in 1947 was enlarged to include three formulations of 2-heptadecyl-glyoxalidine. The 341-A was a dry wettable powder containing 40 per cent toxicant plus a wetting agent. The 341-B was a

TABLE 2.—*Cooperative tests of 341 slurry in 1947*

County	Material[a]	Percentage of leaf spot[b]		
		July 9–10	August 7	October 17–18
Niagara	341, 3 qt.	6.1*	.....	53.5
	Cupro K	10.8	.....	41.9
Orleans	341, 3 qt.	5.1**	17.4*	23.4
	Cupro K	10.2	25.3	41.4
Monroe	341, 3 qt.	27.8*	85.9*	94.0
	Cupro K	48.1	98.7	99.7
Average	341	13.0**	51.6	57.0
	Cupro K	23.0	62.0	61.0

[a] One gal. of the 1947 slurry contained 1.54 lb. toxicant. The 3-qt. dosage gave 1.16 lb. toxicant per 100 gal.
[b] * Probably significant (5 per cent level); ** highly significant (1 per cent level).

similar dry dispersible powder containing 33 per cent toxicant. The 341-C was a solution containing 54 per cent toxicant.

Since this was a grower-sprayed test, single rows of 24 trees for each treatment were randomized in each of two plots. Formulations A and B were applied for the first three sprays followed by two low-soluble copper sprays. Two additional rows received five sprays of formulation B; and four additional rows received five sprays of low-soluble copper. It was planned to apply the 341-C solution in the first three sprays, but fruit injury in the form of pitting was so severe after the first application that the dosage was cut in half for the second spray and copper was used in the three sprays following. Formulations A and B were applied at a dosage to give $\frac{3}{4}$ lb. toxicant per 100 gal. Formulation C contained $\frac{1}{2}$ lb. toxicant per 100 gal. in the first-spray and $\frac{1}{4}$ lb. in the second application. Severe pitting (30 per cent of the fruits) was caused by solution C but not by the A or B form of glyoxalidine or by the copper. Traces of fruit injury were still present at harvest.

TABLE 3.—*Cooperative test of 341 formulations, Boller Farm, Wayne County, New York, 1947*

Date	Percentage leaf spot				
	A (3)[a]	C (2)	B (3)	B (5)	Cu (5)
June 12	23.2	7.7	12.9	12.7	11.8
July 12	31.0	23.2	27.8	21.7	30.9
August 6[b]	74.7	65.0	63.0	41.9	75.1
October 16	89.1	79.4	80.0	77.5	84.1

[a] Numbers in parentheses refer to number of sprays.

[b] Leaf-spot control in August was significantly (5 per cent) better with five sprays of 341-B than with three sprays of 341-A or five sprays of copper.

The 341-A was less effective in leaf-spot control (Table 3) than the other glyoxalidine formulations. Although the 341-C was effective, the injury factor eliminates this formulation. As in the two previous years, no significant differences were evident in the October counts. The value of October counts seems questionable, from the viewpoint of both practical importance and accuracy, in determining the relative effectiveness of the fungicide.

In December a number of the terminals used for summer leaf counts were removed from the southeast and southwest sides of five trees receiving five sprays of 341-B and five trees receiving five sprays of low-soluble copper. The terminals of the 341-sprayed trees were longer and averaged more buds per terminal, and the buds were about 7 per cent greater in diameter, than on the trees receiving sprays of low-soluble copper. The terminals were not gathered in all blocks, so that no measure of significance is possible; but the data suggested a lower phytotoxicity for 341-B than for the low-soluble coppers. The grower reported higher yields in 1948 from the two rows receiving 341-B throughout 1947 than from the copper-

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sprayed rows; this appeared to substantiate the incomplete bud counts of the previous December.

In 1948 a microfine 341-B (3–5 μ) was tested in comparison with the coarser 341-B (18–20 μ) commercially available in that year. Both were used at several dosages, with and without the addition of 1 pt. self-emulsifying cottonseed oil per 100 gal. Fermate was used with and without the oil sticker. Cupro K with oil sticker was also included. One dosage of a purified form of the C solution called 341-SC was also tested. In all, 12 treatments, replicated six times, were randomized in a modified Latin square giving six blocks north and south and six pairs of rows east and west. Each plot consisted of three trees which had no symptoms, during 1945 or the three previous years, of cherry yellows or ring spot. Sprays were applied by the writers. Because of the very low incidence of leaf spot in 1947, no significant differences in leaf-spot control were obtained.

TABLE 4.—*Dropped and yellow leaves on October 26, 1949, at the Horn Farm, Wayne County, New York*

Fungicide[a]	Equivalent angle	Percentage
Fine B, ¾ lb.	59.40	11.5
Do ¾ lb. + oil	71.47	16.3
Do ½ lb.	75.50	18.1
Do ¼ lb.	72.45	16.7
Coarse B, ¾ lb.	66.75	14.3
Do ¾ lb. + oil	71.95	16.5
Do ½ lb.	78.48	19.4
SC, ½ lb., 2 pt.	61.57	12.3
Cupro K, 3 lb.	84.42	22.2
Fermate, 1½ lb.	68.78	15.0
Do 1½ lb. + oil	66.72	14.3
LSD (5 per cent level)	14.45	

[a] The dosages for 341 are in lb. of toxicant per 100 gal.

The limited data suggested that 341-B in the finely divided form was more effective than in the coarser form, and would probably be effective at lower dosages than were previously recommended. Dropped leaves, yellow leaves, and spotted leaves were counted at intervals through the summer. No significant difference in the number of leaves yellowed and dropped appeared until October. These data are in table 4.

While there were no significant differences in leaf-spot control between the coarse and fine 341-B, the data suggest that the finer form would be more effective in a more typical leaf-spot year. The addition of summer oil gave no measurable difference in the yellow and dropped leaves with either 341-B or Fermate. The more finely divided B seemed to warrant further testing at low dosages, and the SC solution seemed worthy of further trial. The finely divided 341-B was more easily mixed with water under agitation in the spray tank and, since injury was not increased by

air-grinding the product, the fine material was made commercially available in 1949.

In 1949 the test was conducted in an orchard in Orange County in which there had been a high percentage of leaf spot in 1948. The 341-B (referred to as Fine B in table 4) was used at 2, 1½, 1, and ½ lb., giving ⅔, ½, ⅓, and ⅙ lb. of toxicant per 100 gal. respectively. An experimental formulation, 341-VG, was used at 1 lb. and ½ lb. giving ¼ and ⅛ lb. toxicant per 100 gal.

The 341-C, which is the solution sold for use on apples, was presumed to be less phytotoxic than the solution causing fruit injury in 1946. It was used at 1½ pt. and ¾ pt., giving slightly more than ½ lb. and ¼ lb. of toxicant per 100 gal., respectively. The highly purified solution, 341-SC, was used at 2 pt. or ½ lb. toxicant per 100 gal.

TABLE 5.—*Equivalent angle and percentage leaf spot and leaf drop at various dates. Beckman Farm, Orange County, N. Y., 1949*

Fungicide[a]	June 3		June 23–25		September 6	
	Angle	Per cent	Angle	Per cent	Angle	Per cent
B, ⅔	2.72	0.2	12.61	4.8	22.70	14.9
B, ½	2.86	0.2	14.56	6.3	24.61	17.3
B, ⅓	6.78	1.4	21.24	13.1	27.68	21.6
B, ⅙	12.85	4.9	23.91	16.4	37.11	36.4
SC, ½	5.69	1.0	18.09	9.6	23.41	15.8
C, ½	11.19	3.8	22.52	14.7	26.55	20.0
C, ¼	10.86	3.6	27.55	21.4	31.95	28.0
VG, ¼	9.34	2.6	19.55	11.1	36.56	35.5
VG, ⅛	7.18	1.6	19.44	11.1	37.41	36.9
Cupro K, 3	9.98	3.0	25.39	18.4	42.21	45.2
Fermate, 1½	8.94	2.4	19.35	11.0	25.71	18.8
Check	19.98	11.7	28.38	22.6	54.68	66.6
LSD, 5 per cent	5.17		6.37		11.02	
1 per cent	6.86		8.46		14.60	

[a] B, SC, C, and VG refer to formulations of 341. Numbers refer to pounds of toxicant per 100 gal. for formulations of 341.

Three pounds lead arsenate and 3 lb. hydrated lime were added to all sprays before harvest, except Fermate. One lb. lime was added to all the 341 formulae in the after-picking spray. For comparison, Fermate at 1½ lb. and Cupro K at 3 lb. plus 1 pt. self-emulsifying cottonseed oil and 3 lb. lime were used.

The check trees received 3 lb. each of lead arsenate and lime in all sprays before harvest. All treatments were on single trees in eight randomized replicates.

While leaf spot was low on all sprayed plots, the trees receiving 341-B at 2 lb. and 1½ lb. (⅔ lb. and ½ lb. toxicant) had the least leaf spot (Table 5). On June 3 these two treatments were significantly superior (1 per cent level) to the C formulations, the lowest dosage of B (½ lb. con-

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taining $\frac{1}{6}$ lb. of toxicant), and check. On the second count, June 23–25, the trees receiving formulation B at $\frac{2}{3}$ and $\frac{1}{2}$ lb. of toxicant still had the least leaf spot and leaf drop. On September 6 the trees receiving the SC solution with $\frac{1}{2}$ lb. toxicant had about the same amount of leaf spot and leaf drop as trees receiving formulation B at $\frac{2}{3}$ and $\frac{1}{2}$ lb. toxicant. On all three counts trees receiving $1\frac{1}{2}$ lb. Fermate had slightly more leaf spot than trees sprayed with these three materials. The difference on June 3 between Fermate and B at $\frac{2}{3}$ and $\frac{1}{2}$ lb. was significant (5 per cent level). On June 23–25 only the $\frac{2}{3}$ lb. of 341-B was significant (5 per cent level) over Fermate, and on September 6 there was no significant difference. The solutions 341-C and 341-SC appeared relatively better in leaf-spot control in September than in June. In the September counts no significant dif-

TABLE 6.—*Equivalent angles and percentage of leaf spot on remaining leaves and of leaves dropped, September 6, 1949*

Fungicides[a]	Leaf spot on remaining leaves		Total leaf drop	
	Angle	Per cent	Angle	Per cent
B, $\frac{2}{3}$	20.38	12.1	7.55	1.7
B, $\frac{1}{2}$	19.80	11.5	11.95	4.3
B, $\frac{1}{3}$	22.99	15.2	15.52	7.2
B, $\frac{1}{6}$	32.40	28.7	16.99	8.5
SC, $\frac{1}{2}$	18.55	10.4	9.94	3.0
C, $\frac{1}{2}$	23.55	15.7	10.10	3.4
C, $\frac{1}{4}$	27.32	21.1	15.61	7.2
VG, $\frac{1}{4}$	32.26	28.5	16.31	7.9
VG, $\frac{1}{8}$	32.54	28.9	17.30	8.8
Cupro K, 3	39.75	40.9	17.78	9.3
Fermate, $1\frac{1}{2}$	23.36	15.7	8.54	2.2
Check	50.61	59.7	25.05	17.9
LSD, 5 per cent level	8.93		7.17	
1 per cent level	11.84		8.07	

[a] B, SC, C, and VG refer to formulations of 341. Numbers refer to lb. of toxicant per 100 gal. for 341 formulations, and to total lb. of the commercial fungicides Cupro K and Fermate per 100 gal.

ferences appeared in leaf spot and leaf drop between trees sprayed with 341-B at $\frac{2}{3}$, $\frac{1}{2}$, and $\frac{1}{3}$ lb. toxicant, 341-SC at $\frac{1}{2}$ lb. toxicant, 341-C at $\frac{1}{2}$ lb. and $\frac{1}{4}$ lb. toxicant, and Fermate. By this date all these materials were highly significant (1 per cent level) over Cupro K and the checks that received only lead arsenate and lime.

A number of workers have expressed their data on cherry fungicides in terms of percentage defoliation and percentage of remaining leaves showing leaf spot. Data in this form for September 6 are in table 6.

In Table 7 the sugar content of cherries sprayed with various fungicides is expressed as percentage of solids in solution, obtained by refractometer readings of the expressed juice of the fruit. No significant difference in the size of the cherries was found in 1949 to explain the significant

differences in percentage of solids in solution in the juice of cherries receiving the various fungicides. The dosages in Table 7 are expressed in pounds of actual toxicant for the 341 formulations and in total pounds of Cupro K and Fermate in 100 gal. The average percentage of solids of the cherries sprayed with all dosages of the powdered forms 341-B and of 341-VG showed a significant (1 per cent) increase in sugar content over cherries receiving the solution form 341-C at $\frac{1}{2}$ lb. toxicant, and over Cupro K and Fermate. Cherries receiving 341-B at $\frac{2}{3}$ lb. toxicant were significantly (1 per cent) higher in sugar content than those receiving $\frac{1}{2}$ lb. dosage of 341-C, probably due to the severe leaf injury which occurred with the 341-C

TABLE 7.—*Refractometer readings of percentage of solids in solution in expressed juice of cherries receiving different fungicide treatments, July 3, 1949*

Fungicide[a]	Percentage solids	Fungicide[a]	Percentage solids
B, $\frac{2}{3}$	17.32	VG, $\frac{1}{4}$	16.72
B, $\frac{1}{2}$	16.46	VG, $\frac{1}{8}$	16.51
B, $\frac{1}{3}$	16.91		16.62 (average for 16 replicates)
B, $\frac{1}{6}$	16.68		
	16.84 (average for 32 replicates)	SC, $\frac{1}{2}$	16.01
		Cupro K, 3	15.18
C, $\frac{1}{2}$	15.35	Fermate, $1\frac{1}{2}$	15.06
C, $\frac{1}{4}$	16.86		
	15.61 (average for 16 replicates)	Check	16.02

	8×8	8×16	8×32	16×16	16×32
LSD, 5 per cent level	9.93	0.81	0.74	0.66	0.57
1 per cent level	1.23	1.07	0.98	0.88	0.76

[a] B, SC, C, and VG refer to formulations of 341. Numbers refer to lb. of toxicant per 100 gal. for 341 formulations and total lb. of the commercial fungicides Cupro K and Fermate per 100 gal.

solution. Cherries receiving 341-B at the $\frac{2}{3}$-lb. dosage were also significantly (1 per cent) higher in sugar content than the check trees, and the check trees were significantly (5 per cent) higher in sugar content than the cherries receiving Fermate. The cherries receiving the 341-SC solution were also significantly (5 per cent) higher than those sprayed with Fermate.

Lewis and Groves (4) have shown the solids in solution to be correlated with the drained weight of canned cherries, so that the lower sugar content of cherries sprayed with Fermate and with Cupro K may be of economic importance. Delay in maturity caused by these fungicides is the probable explanation for the lower sugar content. Previous experiments of the authors and others have not shown a reduction in sugar content.

SUMMARY AND CONCLUSIONS

In two years of grower-sprayed tests and two additional years of tests in which spraying was done by the writers, 2-heptadecyl-glyoxalidine (341) has given outstanding control of cherry leaf spot. A slurry form was used

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in 1946 and 1947 and two powdered forms and a solution in 1948. One of the dry wettable powders, 341-B, gave excellent leaf-spot control; the other was inferior. The solution 341-C was highly effective in leaf-spot control but caused severe fruit injury. Limited evidence indicated that 341-B was less injurious to the trees than the low-soluble copper, Cupro K. In 1948 a more finely divided form of 341-B than the commercial grade was tested and found to be no more phytocidal, probably more effective against leaf spot, and easier to mix in the tank. This more finely divided 341-B was used in 1949 at various dosages and found more effective in leaf-spot control than Fermate and low-soluble copper at dosages of 1½ lb. and 2 lb. of the commercial product. The fruit on trees sprayed with the 341-B were highest in solids in solution, in 1949 tests.

In all four years of these tests the sprays were applied at the dates recommended by the Spray Information Service. Glyoxalidine is an excellent protectant but does not possess the eradicative properties of the low-soluble copper sprays. If the sprays are not properly timed on a protective schedule, the copper sprays would be expected to give better leaf-spot control than the glyoxalidines.

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PHYTOPATHOLOGICAL NOTES

A Method of Testing Some Plants for Curly Top. N. J. GIDDINGS. In connection with studies of the curly-top disease it is necessary to test many plants to determine whether that virus can be recovered from them. If the entire plant is available, in a growing condition, it is usually quite easy to cover some portion of it with a cage, place nonviruliferous sugar-beet leafhoppers in the cage for a few days, and then transfer them to small, susceptible, sugar-beet plants to determine whether they have picked up curly-top virus from the suspected plant. When specimens of plants are collected from the field or are shipped in to be tested the problem is more difficult, since portions of vine such as cucurbits or tomato may not arrive in good fresh condition. The pieces of vine must be placed in water and they are not likely to recover if they have wilted. Leafhoppers will not live long or feed well on the plant tissue unless it is comparatively fresh and unwilted, and they do not survive well on normal portions of melon or squash vine.

While testing some of the curly-top virus strains on watermelon it occurred to me that possibly the leafhoppers would survive well on portions of the fruit and that they might pick up the virus from it. Blocks of tissue cut to include the rind and some of the adjacent inner tissue were carefully covered with Baudruch Capping Skins[1] and placed in a cage, and nonviruliferous leafhoppers were allowed to feed upon them. These leafhoppers survived better than similar ones fed upon portions of vine and readily infected sugar-beet test plants.

Tests thus far have indicated that there was much better survival if the leafhoppers were fed on tissue that was not fully ripe, and that they picked up the curly-top virus just as readily from such tissue as from any that was fully ripe. Specimens of muskmelon fruit sent from curly-top-suspected vines in Arizona were found to be very good for determining the presence of the virus. The fully ripe muskmelons were not satisfactory because the juice came through the membrane and the leafhoppers soon became entangled in it and died.

Experiments with tomato fruits have shown that they may be handled in a similar manner and that curly-top virus can readily be recovered from fruits taken from infected vines. Tomato fruits are usually small, as compared to melon fruits, and the cut surface dries quite rapidly even through the membrane, so that there was some difficulty because of drying. The leafhoppers were usually allowed to feed on the fruit for 2 or 3 days at room temperature.

It has also been found that good results can be obtained by squeezing the juice from a small amount of tomato pulp through fine cloth, filtering it through ordinary filter paper, adding sucrose solution to make it about

[1] Carter, Walter. An improvement in the technique for feeding homopterous insects. Phytopath. 18: 246–247. 1928.

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5 per cent sugar, and using drops of this material on feeding membranes at high temperature, as described by Bennett.[2] A small amount of the pulp placed directly on the under side of the feeding membrane also gave positive results, but seemed less satisfactory than the filtered juice with added sugar. It is probable that either the juice or the pulp method would be quite satisfactory with melons and, presumably, there would be no need to add sugar.

Fruits can usually be shipped or carried more easily than portions of vine, and the fruits may be placed in storage for some time if it is not convenient to test them at once.—Division of Sugar Plant Investigations, U. S. Department of Agriculture, Riverside, California.

The Use of Thin Polythene Sleeving for Moist Chambers in Inoculation Studies. W. R. STANTON. In the study of "strain × variety" interaction using the pear scab fungus (*Venturia pirina* Aderh.), it was desired to enclose separate shoots of pot trees in moist chambers. Glass and rigid plastic cylinders were unsuitable for the numbers of inoculations involved, as they require strong supports and present difficulties in sealing.

Flexible plastics usually require an internal former to prevent undue contact between the foliage and the plastic film. A plastic sleeve is, however, light and the two ends can be easily crimped to form a closed chamber. A plastic film for this use should be 1) resistant to tearing, 2) waterproof and impervious to water vapor, 3) chemically inert (not liable to fungus attack and not a nutrient for fungal growth), 4) free from volatile compounds (including solvents) which might affect the infection picture, and 5) translucent (for most purposes).

Messrs. Imperial Chemical Industries (Plastics Division) suggested that polythene (polymerized ethylene) film might be suitable and supplied some 5-in.-diameter sleeving made by heat-sealing strips of the 0.004-in.-thick sheet. Sleeving of this type has been tested in 1948 and 1949 under field and greenhouse conditions. The original sleeves were supported by a spiral of 1/10-in.-diameter aluminium rod which was continued below the sleeve as a straight rod for fastening to the branch. Top and bottom of the sleeve were closed with aluminium wire. A plug of cotton wool around the branch at the lower closure permitted gaseous exchange.

The sleeves were capable of being washed with strong detergents and sterilized chemically with sodium hypochlorite.

More recently a proprietary brand of polythene extruded in tube form has been used and it is found that this material used at 4-in. diameter and 0.0015 in. thick is suitable for greenhouse studies. The sleeve is sufficiently light to render support for the branch unnecessary and, being very resistant to wetting, is not pulled by water droplets into contact with the moist leaves. It is sufficiently rigid to retain its tube form through the major portion of

[2] Bennett, C. W. Studies on properties of the curly-top virus. Jour. Agr. Res. [U.S.] 50: 211–212. 1935.

its length. At larger diameters, however, an internal spiral of aluminium is still desirable. These two types of chamber are illustrated (Fig. 1).

As the conidiospores of *Venturia pirina* germinate in water droplets on the leaf, they were sprayed on to the leaves in an aqueous suspension. The humidity was initially raised by atomizing water into the interior of the chamber and was maintained subsequently by the transpiration of the leaves. —John Innes Horticulture Institution, Bayfordbury, Hertford, Herts., England.

FIG. 1. Left: Sleeving, 4 in. diameter, 0.0015 in. thick, used without former to enclose an extension shoot of a 2-year-old pear. Right: Sleeving, 9 in. diameter, 0.0015 in. thick, used with a spiral former of aluminium to enclose a fruit cluster on an older tree.

Storage Temperature and the Development of Internal Cork in Sweet Potatoes. MARGUERITE S. WILCOX AND BOYCE D. EZELL.[1] Nusbaum[2] described internal cork of sweet potatoes in November 1946, and by early 1950[3] the disease had been reported in Maryland, Virginia, North Carolina, South Carolina, Georgia, Alabama, Mississippi, Louisiana, Tennessee, and Texas. Martin[4] reported that in three lots of sweet potatoes with 19.4 per cent infection at harvest the internal cork had increased to 80 per cent after 5 months in storage. Nusbaum[3] also reported that the incidence of internal

[1] Division of Fruit and Vegetable Crops and Diseases, Agricultural Research Administration, Bureau of Plant Industry, Soils, and Agricultural Engineering, U. S. Department of Agriculture, Beltsville, Maryland.

[2] Nusbaum, C. J. Internal cork, a new disease of sweet potato of unidentified cause. Phytopath. 36: 18–23. 1946.

[3] Nusbaum, C. J. Internal cork of sweet potatoes. South Carolina Agr. Exp. Sta. Bul. 381. 1950.

[4] Martin, W. J. Internal cork of sweet potatoes. Research in Agriculture. Annual Report, La. Agr. Exp. Sta., 1947–1948. P. 98. 1949.

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cork increases during storage. The effect of storage temperature on the development of the disorder apparently has not been reported.

During some compositional studies of sweet potatoes we had opportunity to observe the effect of storage temperature on internal cork development when it was found that we had unknowingly used Porto Rico roots affected by this disorder. In the work being done 50 or more roots were split lengthwise at harvest, shortly after curing, and at 4- to 6-week intervals during storage. Using one half of the root, cross sections were removed with a cork borer from the central portion; the other half was split lengthwise one or more times, depending on size, and ground in a food chopper. The methods used, while not designed for internal cork studies, did afford a fair chance of detecting most of the affected roots. When the above method was tested on a lot of 50 roots late in the storage season, 26 per cent internal cork was detected; by slicing the same roots crosswise in $\frac{1}{4}$-in. sections, 30 per cent internal cork was detected. No internal cork was observed at harvest, shortly after curing, or early in the storage period.

TABLE 1.—*Percentage of sweet potatoes developing internal cork when stored at different temperatures*

Storage temperature, °F.	Date examined	No. of roots examined	Percentage of roots affected
At harvest	Oct. 4	50	0.0
70	May 24	142	64.8
60	June 6	54	7.4
60	July 11	55	9.1
55	June 13	54	7.4
55	July 26	56	10.7

On April 17 in several roots stored at 70° F. since October 12 and otherwise in good condition, numerous brownish areas were observed throughout the flesh, apparently unconnected with the surface. This disorder was identified as internal cork.[5] Although some nematode injury was present, it was always easily distinguished from internal cork. At lower temperatures fewer roots were affected than at 70° F. At the next examination, on May 24, both the number of affected roots and severity of infection had greatly increased in the 70° storage lot. Many of the spots had coalesced to form large corky areas in the roots. Because sound roots were desired for the work being done, a correspondingly greater number of roots was examined as the infection increased. The observations on internal cork in roots stored for several months at 70°, 60°, and 55° F. are in table 1. Comparable roots stored at 50° F. had decayed from other causes prior to these examinations. The highest percentage of roots were affected and the largest lesions developed in the roots stored at 70° F.

While 70° F. is a higher temperature than is generally recommended for sweet-potato storage, the results reported here would indicate that it is

[5] Diagnosis confirmed by Drs. H. T. Cook and T. T. Ayers.

better for appraising internal cork susceptibility and latent infection than the temperature usually recommended for sweet-potato storage.—Plant Industry Station, Beltsville, Maryland.

Indexing Hosts for Sour-Cherry Ring-Spot and Yellows[1]. H. C. YOUNG, JR. The use of *Prunus tomentosa* for indicating the presence of the necrotic ring-spot virus in sour-cherry buds has been reported by Fink[2]. He compared seedlings of *P. tomentosa* and *P. persica* as indexing plants for the necrotic ring-spot virus and found *P. tomentosa* to be the more efficient one. The reaction of *P. tomentosa* to the cherry-yellows virus complex has not been reported, but would be of importance if this species were used in a cherry budwood indexing program. The following indexing tests confirm the results reported by Fink and show that *P. tomentosa* is an excellent host for detection of the necrotic ring-spot virus, but that the reaction of this species to inoculation with the cherry-yellows virus complex is not diagnostic. The tests also compared *P. cistena* and three peach varieties as indexing hosts for these two cherry viruses.

The comparative tests were made in the greenhouse with 1-year-old *P. tomentosa* on peach roots, 1-year-old *P. cistena* on peach roots, and Carman, J. H. Hale, and Rochester varieties of peach, *P. persica*. Twenty-four trees of each of the five varieties and species in the test, 120 trees in all, were potted and held in common storage from November, 1949, until the latter part of January, 1950, when they were moved into the greenhouse.

A group of 30 trees consisting of six each of the three peach varieties and two Prunus species was used for each separate treatment. On January 25, dormant buds from sour-cherry trees known to contain the necrotic ring-spot virus were chip-budded[3] into the 30 trees of one group and, similarly, dormant buds from sour-cherry trees known to contain the cherry-yellows virus complex were budded into a second group of trees. For controls a third group was budded from sour-cherry trees known to be free from both necrotic ring-spot and the cherry-yellows virus complex; a fourth group was not budded.

Following the budding operation all of the potted trees were held at 40° F. for 2 weeks, then at 75° F. for the duration of the experiment. The low temperature for 2 weeks after budding enhanced materially the expression of mottle, ring patterns, and rosette in peach, and mottle and ring patterns in *P. tomentosa*.

Symptom expression was recorded at monthly intervals until April 28, 1950.

All of the *P. tomentosa* trees budded with the necrotic ring-spot material had strong symptoms by March 25, and the first set of leaves, after showing

[1] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 833, August 16, 1950.

[2] Fink, Harry C. Prunus tomentosa as an index plant for necrotic ring-spot of sour cherry. (Abstr.) Phytopath. 40: 9. 1950.

[3] Talbert, T. J. Grafting and budding fruit trees. Mo. Agr. Ext. Service Circ. 206. August, 1928.

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severe necrotic spots and rings, had been killed. A second set of leaves expanded later, but had no symptoms of virus infection. Some of the *P. tomentosa* trees budded with the yellows virus complex had a faint mosaic or mottle on several leaves, but these symptoms were not sufficiently clear or consistent to be of value in determining the presence or absence of the cherry-yellows virus complex in an unknown budwood sample. This test indicates that the usefulness of this plant in a cherry budwood indexing program would be limited to detection of the necrotic ring-spot virus alone. There were no symptoms on either the unbudded trees or on those budded with healthy buds.

No symptoms of virus infection were observed on any of the *Prunus cistena* trees. However, shoots arising from the peach roots under trees inoculated with the necrotic ring-spot virus bore leaves with severe mottle and ring patterns, indicating that at least the necrotic ring-spot virus had been transmitted through *P. cistena* into the peach roots.

Shoots arising from the peach roots under trees inoculated with the yellows virus complex bore leaves with mottle and ring patterns, but had none of the rosette symptoms normally associated with yellows infection of seedling peach. It has been assumed by many that the mottle and ring patterns observed on peach following inoculation with the yellows virus complex were due to the ring-spot component of that complex. If such is the case, it appears that *P. cistena* has transmitted only the ring-spot component to its peach root. No attempt was made to recover the yellows virus complex from these shoots. Shoots arising from the roots of unbudded trees, or those budded with healthy cherry buds, appeared healthy throughout the study.

Prunus persica, variety Carman, showed strong symptoms following inoculation with the necrotic ring-spot virus (mottle and ring patterns) or with the yellows virus (mottle, ring patterns, and rosette). The variety Rochester, contrary to the report of Berkeley,[4] and the variety J. H. Hale were erratic in symptom expression. Some trees had no symptoms at all, others only a faint mottle on a few leaves. In no case did the yellows virus complex inoculation give rise to a rosette condition in these latter two varieties. In a similar test the previous year, the varieties Redhaven and Halehaven also were rather poor indexing plants, but the variety Triogem, like Carman, showed excellent and characteristic symptoms following inoculation with either the necrotic ring-spot virus or the yellows virus complex. In the field during the spring and summer of 1949, however, when Triogem was inoculated with the yellows virus, no rosette symptoms became evident, which indicated that environmental factors may influence the expression of rosetting. Carman has not yet been tested in the field.—New York State Agricultural Experiment Station, Geneva, New York.

[4] Berkeley, G. H. Cherry yellows and necrotic ring spot of sour cherry in Ontario. No. 1. The value of Prunus persica and P. domestica var. Italian Prune as index hosts. (Abstr.) Phytopath. 37: 2. 1947.

A Ground Glass Homogenizer for Preparing Virus Samples from Plant Tissue. WILLIAM N. TAKAHASHI. A tapered, self-fitting, ground glass homogenizer of simple design has been useful in the preparation of virus

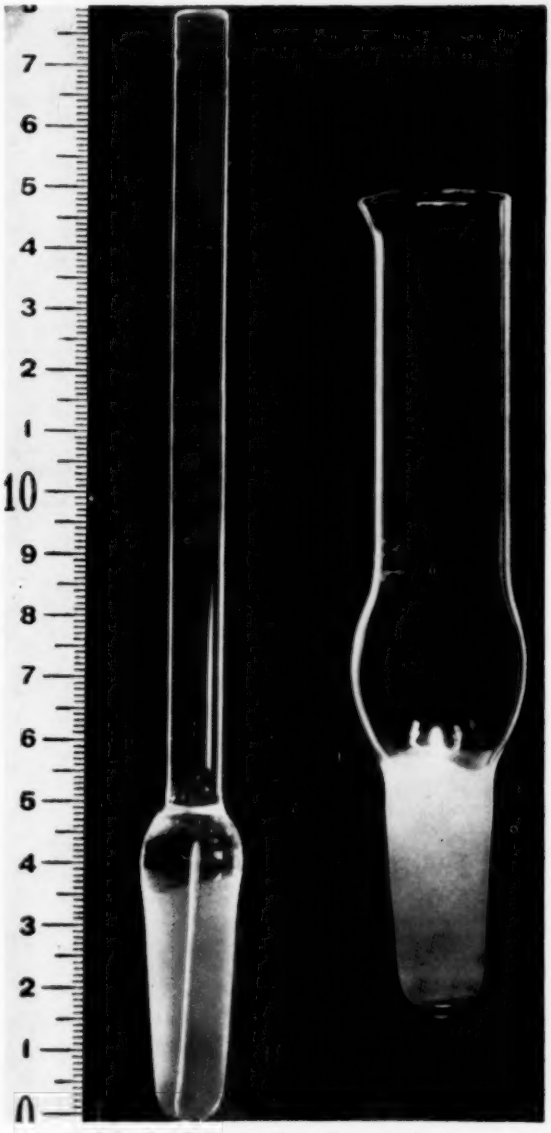

FIG. 1. Ground glass homogenizer.

samples from either fresh or frozen plant tissue.[1, 2, 3, 4, 5] It is particularly suited for preparing inoculum from small pieces of tissue.

The two members, the mortar and the pestle, are shown in figure 1. The line appearing on the grinding surface of the pestle is actually a continuous groove which extends from the shoulder around the base and up

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to the shoulder on the opposite side. Two such grooves cut in each pestle, crossing on its base, serve to supply liquid to the grinding surface, and in addition act as gross cutting edges. To prevent the compacting of unhomogenized tissue at the bottom of the homogenizer it is desirable that the base of the pestle be rounded and fit snugly into the rounded bottom of the mortar cavity. The pestle may be operated manually or by a cone drive motor. The bulge on the mortar serves as a well into which the homogenate may be collected and there be reached easily with a cotton-tipped applicator stick.

When necessary the cutting surfaces may be sharpened and at the same time fitted by grinding with a suspension of fine emery powder.—Division of Plant Pathology, University of California, Berkeley, California.

[1] Takahashi, William N., and T. E. Rawlins. An electron microscope study of two strains of potato X virus. Amer. Jour. Bot. 33: 740–742. 1946.

[2] Takahashi, William N., and T. E. Rawlins. An electron microscope study of tobacco mosaic virus extracted from pulp and juice after various periods of infection. Phytopath. 38: 279–282. 1948.

[3] Takahashi, William N. The morphology and host range of *Brassica nigra* virus. Amer. Jour. Bot. 36: 533–535. 1949.

[4] Takahashi, William N., and T. E. Rawlins. The length of tobacco mosaic virus particles from juice and from leaf residue. Phytopath. 39: 672–675. 1949.

[5] Takahashi, William N. The effect of pH on the linear aggregation of tobacco mosaic virus. Amer. Jour. Bot. 36: 642–645. 1949.